New lessons from an “old” drug:

A proteomic study of anthracycline-induced apoptosis in acute myelogenous leukemia

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Abbreviations

2DE	Two-dimensional polyacrylamide gel electrophoresis
5`TOP	5`terminal tracts of pyrimidines
AraC	Cytarabine, cytosine β-D-arabinofuranoside
ATRA	All-trans retinoic acid
BNML	Brown Norwegian rat Acute Myeloid Leukemia
CALM	Clathrin Assembly Lymphoid Myeloid leukemia gene
CCV	Clathrin coated vesicles
CHX	Cycloheximide
CKII	Casein kinase II
CLC	Clathrin light chain
CME	Clathrin mediated endocytosis
CML	Chronic myeloid leukemia
COFRADIC	Combined fractional diagonal chromatography
DISC	Death-inducing signaling complex
DNR	Daunorubicin
EGFR	Epidermal growth factor receptor
ER	Endoplasmatic reticulum
FADD	Fas-associated death domain
FAB	French-American-British classification
GA	Geldanamycin
GM-CSF	Granulocyte macrophage colony stimulating factor
Hsp90	Heat shock protein 90
IDA	Idarubicin
IRES	Internal ribosome entry site
MS	Mass spectrometry
PABP	Poly-A binding protein
PGE2	Prostaglandin E2
PKR	Protein kinase R
ROS	Radical oxygen species
RTK	Receptor tyrosine kinase
SCF	Stem cell factor, ligand for c-kit
SILAC	Stable isotope labeling with amino acids in cell culture
SNARE	Soluble N-ethylmaleimide-sensitive factor-attachment protein receptor
Tf	Transferrin
VAMP	Vesicle-associated membrane protein

Summary of Thesis

Anthracyclines have been first line drugs for more than 3 decades in the treatment of acute myelogenous leukemia (AML). Even though the mechanism of action of anthracyclines has been extensively studied over the years, large gaps remain in our understanding of why they act better than most other drugs and how they induce cancer cell death. The present study was undertaken to identify protein changes that could shed further light on how the anthracyclines induce and execute apoptosis. By this approach, we hoped to identify proteins or pathways of particular importance in AML cell death that in the future could be exploited as new drug-targets and lead to the development and use of less toxic or more efficient drugs than the anthracyclines. We also expected to reveal weaknesses in anthracycline actions that could explain why many patients in the end develop resistance to the drug. Targeting these weak points by other drugs could open new avenues for combination treatment.

By using various proteomic approaches to identify cleavage, degradation or altered phosphorylation of proteins, we found that components associated with the Hsp90 multi-protein complex, clathrin-mediated endocytosis, and protein synthesis to be targeted in cells undergoing or about to undergo anthracycline-induced apoptosis. The Hsp90 complex was targeted through cleavage of its co-chaperone p23. The truncation or downregulation of components of the clathrin-dependent endocytic pathway were associated with decreased endocytosis and increased accumulation of surface receptors with cell death-modulating capability.

We also found that anthracyclines induced the pre-apoptotic synthesis of presumed anti-apoptotic proteins. That the pre-apoptotic protein synthesis served a net survival role was suggested by the ability of protein synthesis inhibitors to enhance anthracycline-induced cell death. This was true both in cell culture and in animal models of AML.

In conclusion, the Thesis contributes to the knowledge of how anthracyclines induce apoptosis by pinpointing new protein targets and pathways that are heavily modified not only in the execution-phase, but also in the induction-phase of apoptosis. Based on our findings, we propose a novel anthracycline-based combination treatment regime for patients with AML that consists of an anthracycline and a protein synthesis inhibitor administered in the pre-apoptotic phase.

List of papers

Paper I:

Gro Gausdal, Bjørn Tore Gjertsen, Kari Espolin Fladmark, Hans Demol, Joël Vandekerckhove and Stein Ove Døskeland: **Caspase-dependent, geldanamycin-enhanced cleavage of co-chaperone p23 in leukemic apoptosis.** Leukemia (2004) 18, 1989–1996.

Paper II:

Gro Gausdal, Bjørn Tore Gjertsen, Emmet McCormack, Petra Van Damme, Randi Hovland, Camilla Krakstad, Øystein Bruserud, Kris Gevaert, Joël Vandekerckhove, and Stein Ove Døskeland: **Abolition of stress-induced protein synthesis sensitizes leukemia cells to anthracycline-induced death.** Manuscript, In revision at Blood.

Paper III

Gro Gausdal, Jørn Skavland, Kris Gevaert, Joël Vandekerckhove, Stein Ove Døskeland and Bjørn Tore Gjertsen: **Clathrin light chain is targeted by anthracyclines and results in blocked endocytosis and attenuated receptor mediated survival signaling in leukemia**, Manuscript.

Introduction

Acute myeloid leukemia

Acute myeloid leukemia (AML) is a group of heterogeneous diseases characterized by accumulation of immature blasts of myeloid precursors in the bone marrow and peripheral blood. This is due to a differentiation block in hematopoietic progenitor cells at an early stage of myelopoiesis combined with proliferation of the immature blasts and eventually leads to suppression of normal bone marrow functions. Common symptoms of AML are diverse and non-specific and includes fatigue, hemorrage, infections and fever, all symptoms resulting from leukemic infiltration of the bone marrow with resultant cytopenia [1].

A major hypothesis for myeloid leukemogenesis, the "two-hit" model, states that mutations both in a transcription factor and a tyrosine kinase are required for development of AML as this would impair cell differentiation and confer survival and/or proliferative advantages [2]. Receptor tyrosine kinases (RTK) like Flt3 and c-kit are reported mutated and constitutive active in a substantial number of AML cases [3,4]. Mutations and chromosomal translocations leading to impaired differentiation and subsequent apoptosis such as PML-RARα and AML1-ETO are also frequent events [5].

Based on cytogenetics, patients are often classified into three major subgroups with different disease outcome. Translocations resulting in the AML1-ETO fusion protein, t(8;21)(q22;q22), often associated with FAB-classification AML-M2, and the PML-RARα fusion protein, t(15;17)(q22;q21), often associated with promyelocytic AML-M3, are both prognostically favourable changes [6]. Changes involving deletions of chromosomes 5 and 7 give rise to adverse prognosis. In addition to chromosomal translocations, aberrations in oncogenes, e.g. the presence of a mutated form of Flt3 [7], and expression of the Bcl-2 family members are prognostic indicators, and the ratio between the anti-apoptotic Bcl-2 and the pro-apoptotic Bax have been shown to predict disease outcome in AML [8].

Treatment

Current standard treatment of AML includes initial induction therapy to achieve hematological remission meaning 5% or less leukemic blasts in the bone marrow (disease is diagnosed upon the presence of 30% blasts in the bone marrow [9]) followed by consolidation treatment [1]. The induction treatment regime typically consists of an anthracycline (daunorubicin, DNR, or idarubicin, IDA) in combination with cytosine arabinoside (AraC). AraC is typically given by continuous infusion while DNR is given as an intravenous infusion for 30 min a day for the three first days of AraC treatment. After remission is achieved, consolidation treatment consisting of high-dose AraC is administered [10]. For elderly patients (>60 years) an alternative to the high dose AraC for consolidation therapy must often be used due to high risk of treatment-related toxicity. For young patients, bone marrow transplantation is often offered as consolidation therapy for patients with intermediate or bad prognosis [11]. Following relapse of the disease, patients are usually treated with additional chemotherapy or stem cell transplantation [12]. This approach is marginally successful, and the overall 5-year survival rate is only 30–40%, and less than 15% in elderly patients.

The anthracyclines have been used successfully for over 40 years in the treatment of AML. However, they are mainly nonspecific in their mechanisms of action and will kill normal cells along with the cancer cells and can lead to severe systemic toxicities. Therefore, over the years, several new treatment modalities with drugs aimed at specific molecular targets have been proposed for AML treatment. The most successful has been the introduction of all-trans retinoic acid (ATRA) in combination with low-dose chemotherapy in treatment of patients with promyelocytic leukaemia, APL (AML-M3) [13,14]. Inhibition of RTKs are though to have an inhibitory effect on proliferation, and specific inhibitors have been developed and clinically tested. The combination of such inhibitors together with conventional therapy are considered to significantly improve clinical outcome in patients with AML [15-18]. Also inhibition of survival signaling from of Ras [19], Hsp90 [20] and mTOR [12] have been tested/postulated for combination therapy in AML. However, the drawback with using drugs that specifically target a kinase or another molecular target is that even though the initial response is successful, the patients

often relapse with a form of the disease that now show resistance toward that drug. This is often the case after treatment with Gleevec, a specific inhibitor of Bcl-abl, used in treatment of CML [21]. Another scheme that has been tried in order to reduce the overall toxicity of the chemotherapeutic drugs, is to ensure specific delivery of the drug to cancer cells by coupling the drugs to monoclonal antibodies specific for antigens/receptors present on cancer cells and utlilize the cells endocytic apparatus to ensure drug uptake [22,23].

Anthracyclines

Anthracyclines are cytotoxic antibiotics that are used in the treatment of hematological and solid neoplasms such as leukemias, lymphomas, breast cancer, prostate cancer and bladder cancer. The anthracyclines have been widely used in cancer therapy due to their ability to induce apoptosis in cancer cells. The anthracycline antibiotics are produced by *Streptomyces coeruleorubidus* or *S. peucetius* and include daunorubicin (DNR), doxorubicin, epirubicin and idarubicin (IDA). DNR and IDA are used in the treatment of AML, and we have utilized them in this study to explore the mechanism of anthracycline-induced apoptosis. IDA is an analog obtained from DNR by removal of the 4-methoxy-group in the D-ring (Figure 1). Compared to DNR, IDA has increased lipophilicity and cellular uptake and has been proposed to be less cardiotoxic [24]. The use of anthracyclines is a double-edged sword. Even though they play an important role in treatment of many cancers, they are cardiotoxic [25] and administration of the drugs induce cardiomyopathy and heart failure refractory to common medication. Due to the damaging effect on cellular DNA, treatment with anthracyclines is also often associated with the induction of secondary cancers.

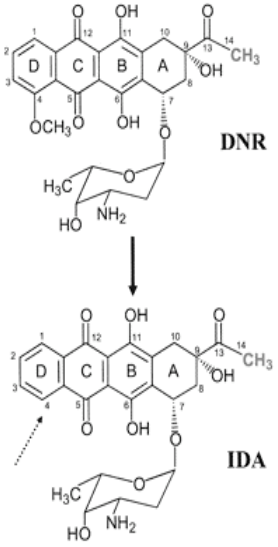


Figure 1. The structure of daunorubicin and idarubicin. Adapted from [24].

Initially, the main cytotoxic mechanism of action for the anthracyclines was thought to be induction of DNA damage. The direct effects of anthracyclines on DNA are DNA double helix distortion due to intercalation and

introduction of DNA breaks. The breaks are introduced due to stabilization of the topoisomerase II complex or reactivity on the DNA backbone by free radicals [24]. In addition to its direct effects on DNA, anthracyclines also have several other effects in leukemic cells such as inhibition of mitochondrial cytochrome C oxidase [26], binding and inhibition of the proteasome [27] and generation of free radical oxygen species (ROS) such as superoxides, peroxides and hydroxyl radicals [28,29]. Even though anthracyclines are, above all, DNA intercalators, which results in genetic damage leading to cell death, increasing evidence suggests that the underlying mechanism for anthracycline cytotoxicity is the induction of apoptosis through intracellular-mediated signaling pathways [30]. It has also been shown that DNA interaction may not be a prerequisite for anthracycline cytotoxicity, so whether drug/DNA interaction is necessary for apoptosis signaling is unknown.

The cellular response to anthracycline treatment is highly regulated by a diverse set of signaling events that includes activation of both pro- and anti-apoptotic pathways such as the sphingomyelin-ceramide pathway, the MAP kinase and stress activated protein c-Jun N-terminal kinase (JNK), the NF-kB pathway and the Fas/FasL system [30]. However the consequence of activation of these signaling pathways are not always obvious. For example, NF-kB activation normally considered an anti-apoptotic effect which may blunt the therapeutic efficiency of drugs, was found to be required for the cytotoxic effect of anthracyclines [31,32]. How these pathways are activated in response to drug is again dependent upon factors such as the presence of oncogenes, tumor supressors and external stimuli such as growth factors. The dependency of the anthracyclines on protein synthesis for their action has been claimed by several studies since co-treatment of cells with a protein synthesis inhibitor inhibited drug-induced apoptosis [33-37]. However, this view has been challenged [38] and particularly when it comes to the dependency of the anthracyclines on induction of the Fas/FasL pathway for their ability to induce cell death [39,40].

Anthracycline-induced death is associated with increased activity of caspases [41], and cell death is abrogated, but not completely blocked by caspase inhibitors [41,42]. Enforced overexpression of the survival proteins Bcl-2 or Bcl-xL protect against anthracycline-

induced apoptosis *in vitro* [43]. In several systems, anthracycline treatment also activates p53-DNA binding and p53 could play an important function in anthracycline cytotoxicity [30].

All these findings implicate that the interactions between the anthracyclines and the cells are extremely complicated and that further studies are required to increase our knowledge about the mechanism of apoptosis induction and execution of the anthracyclines and to elucidate what factors are critical when it comes to resistance development.

Protein synthesis inhibitors

Protein synthesis is an absolute requirement for cellular survival, and several of the antibiotics used to treat bacterial infections (e.g. chloramphenicol, streptomycin, tetracycline, neomycin) function through the inhibition of protein synthesis. Inhibition can be effected at all stages of translation i.e. initiation, elongation and termination. Several toxins that inhibit protein synthesis in eukaryotes are also known, such as cycloheximide, puromycin, emetine, diptheria toxin and ricin. Since the protein synthesis inhibitor utilized the most in this study is cycloheximide, only this toxin will be described in detail.

Cycloheximide (CHX) (from *Streptomyces griseus* [44]) is a wellknown inhibitor of eukaryotic protein synthesis. Although inhibitory effects of CHX have been reported both on initiation, elongation end termination of protein synthesis [45,46], CHX is generally considered an inhibitor of protein elongation. CHX inhibits the translocation of peptidyl-tRNA from the aminoacyl (A) site to the peptidyl (P) site on the ribosome by interacting with the P-site [45].

CHX has for a long time been known to inhibit protein synthesis both in mammalian cell culture systems [47] and in intact mammals [37,48]. Clinically CHX has been used in the treatment of acute cerebral phycomycosis [49] and as an antipyretic drug in patients with Hodgkin`s disease or other malignant neoplasms [50]. In addition, treatment with CHX has been shown to induce involution of psoriatic plaques [51] and to be efficient in the

treatment of superficial basal cell carcinoma of the skin when added topically [52]. Side effects of CHX is blocking of long-time memory [53,54] and acute nausea and drowsiness [50].

Cytotoxic drugs commonly used in cancer therapy promote tumour cell death by inducing apoptosis. As a typical inhibitor of protein synthesis, CHX has been widely used to study the requirement of *de novo* protein synthesis in induction of apoptosis,s and studies have shown that the dependency upon protein synthesis for the ability of different drugs to function efficiently is diverse. The presence of CHX is in most cases essential for Fas- and TNF-α induced apoptosis due to the activation of NF-kB and resulting synthesis of survival proteins [55]. For other drugs such as of etoposide [56,57], camptothecin [58] and doxorubicin [35-37,59] apoptosis was for a long time considered an active process requiring protein synthesis as the presence of CHX inhibited apoptosis. However, protein synthesis does not always seem to be a necessary step in the induction of apoptosis as CHX instead, of preventing epirubicin-induced apoptosis, enhanced the cytotoxic effect of epirubicin [38].

Macromolecular complexes/pathways identified as targets in anthracycline-induced apoptosis

The aim of this Thesis was to identify proteins that were modified either by proteolysis or post-translational modifications in anthracycline-induced cell death in order to learn more about the basic mechanism of action of these drugs and to identify new potential targets for directed therapy. During this study we came across several protein modifications that caught our interest because 1) they were related to macromolecular complexes that are current promising targets in cancer therapy, such as the Hsp90 complex (targeted by geldanamycin, GA) or the protein synthesis machinery (targeted by rapamycin), or because 2) several proteins involved at different stages in the same cellular pathway were targeted at the same time, such as the protein synthesis machinery and clathrin-mediated endocytosis and intracellular trafficking. To give the reader a background for understanding the papers resulting from this work, I will here give a brief general introduction into the three subjects that Paper I-III deals with, namely the Hsp90 multi-protein complex, translational regulation of protein synthesis and clathrin-mediated endocytosis and intracellular trafficking. The introduction will focus on the proteins that

we identified as targets in anthracycline-induced apoptosis and will therefore not be complete when it comes to describing all the proteins and factors involved in and important for the Hsp90 complex, protein synthesis and endocytosis and trafficking.

The Hsp90:p23 multi-protein complex

Hsp90 is an abundant and ubiquitous chaperone expressed in the cells at high levels and necessary for cell viability in eukaryotes (for review see [60]). During times of cellular stress, Hsp90 plays a role in reactivation of damaged proteins [61]. In malignant cells, Hsp90 is found in a constantly active multiprotein complex together with several co-chaperones such as p23 [62], where it acts on a wide range of client proteins (for review, see [63]) and is essential for maturation, stability and activity of numerous signaling molecules involved in cancer progression and proliferation. These client proteins include protein kinases and ligand-regulated transcription factors [64-66] and refs therein).

The Hsp90 co-chaperone p23 is part of the active Hsp90 multi-protein complex and facilitates conformational changes in Hsp90 [63] and stabilizes Hsp90 interactions with transcription factors [67,68] and kinases [69]. p23 also has a passive chaperone activity when it its not bound to Hsp90 and is able to inhibit aggregation of denatured proteins [70,71] and protect against ER stress induced cell death [72]. In addition, p23 has been reported as identical to cytosolic prostaglandin E2 synthase [73].

Recently, three different groups have tried to make knock-out mice of p23 [74-76]. However, even though p23 is dispensable for prenatal development and morphogenesis, p23 seems to be essential for perinatal survival as pups die during the prenatal period. The pups display retarded lung development which is probably due to the lack of a proper glucocorticoid response in p23-/- mice caused by a defect in assembly of a hormone-responsive glucocorticoid receptor-Hsp90 complex that requires p23 for proper function [74,75].

As Hsp90 is involved in binding and stabilization of oncogene proteins, targeting Hsp90 for directed chemotherapy has been very popular during the last decade. The Hsp90 directed drug geldanamycin (GA) and its derivative 17-AAG have been tested with success in several treatment regimes, both alone and in combination with other cytostatic

drugs, and 17-AAG is now in clinical trial [20]. GA is an ATP antagonist that binds to Hsp90 and prevents the association of p23 to Hsp90 [77] since p23 binds to Hsp90 in an ATP-dependent manner [78]. This results in destabilization and proteasomal degradation of Hsp90 client proteins and leads to growth inhibition and apoptosis [79-84]. GA treatment also results in activation of the interferon-induced and double-stranded RNA-dependent protein kinase (PKR) [85]. PKR kinase activation results in protein synthesis inhibition [86] and has been described following apoptosis induction [87].

Protein synthesis: Regulation of translation

Protein synthesis is a highly energy depending process and is therefore exposed to tight regulation under normal conditions, and particularly in stressful situations such as viral infections, hypoxia, nutrient deprivation and exposure to apoptotic stimuli. Regulation of protein synthesis can be exerted on several levels, both by controlling mRNA availability on the transcriptional/processing level and by controlling the translation of proteins on ribosomes. During times of rapid environmental changes, such as acute stress, immediate and selective changes in protein levels are often necessary to ensure a rapid and suitable response. Translational regulation provides the cell with the plasticity that it needs to respond to rapid environmental changes [88].

Translational initiation: cap- versus IRES

The initiation of translation is under most stringent regulation and is a frequent target for translational control through phosphorylational events and proteolytic processing of essential factors. Two different mechanisms of protein synthesis initiation exist, the cap-dependent and cap-independent mechanism. Cap-dependent translation is mediated by the mRNA 5`cap structure ($m^7$GpppN where N is any nucleotide) and is the standard mode of translation used by most cellular mRNAs. 40S ribosomal subunit binding to the 5`capped mRNA is mediated by the eukaryotic translation initiation factor 4F (eIF4F), a complex containing three proteins: eIF4E, the cap-binding subunit; eIF4A, a RNA-dependent ATPase/ATP-dependent RNA helicase; and eIF4G, a scaffold protein for binding eIF4E and eIF4A. In addition binding of eIF4G to the 40S ribosome binding factor eIF3 and the poly(A)-binding protein (PABP) is also required (for review see [89] (Figure 2a).

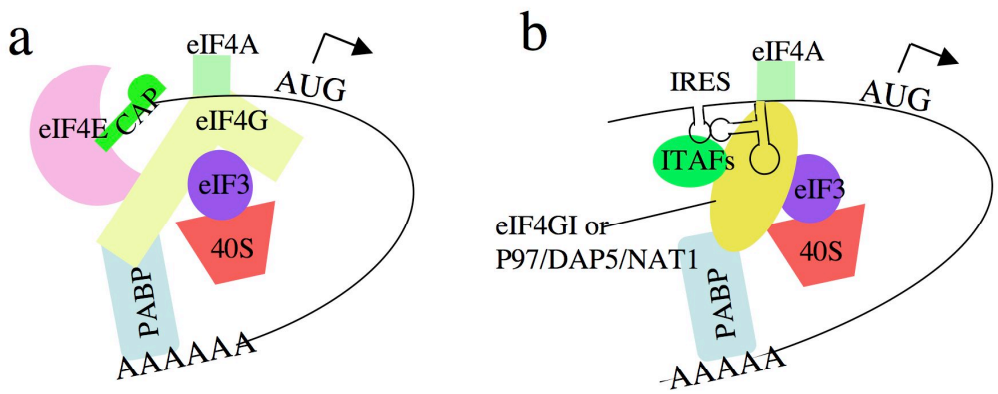


Figure 2. Cap-versus IRES-dependent translation initiation. (a) eIF4E binds to the 5´m^{7}GpppN cap structure. The capped end of mRNA is bridged to the 40S ribosomal subunit by eIF4G which also binds to eIF3. eIF4A is involved in unwinding of the secondary structure of mRNA 5´-UTR. PABP circularizes mRNA through its interaction with both the 3´- and 5´-UTR. (b) ITAFs and proteolytic fragments of eIF4GI or p97/DAP57NAT1 stimulates IRES translation. Adapted from [88].

In cap-independent translation, the mode of translation of many mRNAs under stress, the ribosome is bound to a mRNA structural element called an internal ribosomal entry site (IRES) and requires the same canonical eIFs as cap-dependent, except for eIF4E. The initial steps of recruitment of ribosomes to the IRES are the eIF4A-dependent binding of eIF4G to the IRES with subsequent addition of 40S ribosomal subunit via the eIF4G-eIF3-40S interaction (Figure 2b). Hence, the availability of eIF4E is considered to be a switch between cap-dependent and IRES-mediated translation [90]. eIF4E availability for participation in the eIF4F complex is modulated by a family of repressor molecules, the eIF4E-binding proteins (4E-BPs). The 4E-BPs are substrates of the mammalian target of rapamycin kinase (mTOR), and when hyper-phosphorylated, 4E-BP does not interact with and inhibit eIF4E [91].

mTOR regulates numerous components involved in protein synthesis (for review see [92] and activation of mTOR through the PI3K/Akt pathway as a result of hormonal and nutrient stimulation or oncogene activation, increases cap-dependent translation initiation [93]. In addition to 4E-BP1, mTOR also phosphorylates p70S6K. p70S6K has numerous

substrates among them pro-apoptotic Bad and ribosomal protein S6 [94]. p70S6K was for a long time thought to play a role in up-regulating the translation of mRNAs that contain a oligopyrimidine tract in their 5'-UTR, a 5`TOP, through phosphorylation of ribosomal protein S6 [94-96]. However, this hypothesis has been challenged and p70S6K does not seem to be involved in translationally control of such mRNAs after all [94]. Several mRNAs coding for components of the protein synthesis apparatus contain a 5`TOP considered essential for their regulation at the translational level [97].

Initiation of IRES mediated translation is also regulated at the level of formation of the eIF2-methionyl-initiator tRNA-GTP ternary-initiation complex that is controlled by eIF2-α phosphorylation. Phosphorylation of eIF2-α is mediated by several stress activated kinases such as e.g. PKR, and leads to reduced formation of the eIF2-methionyl-initiator tRNA-GTP ternary-initiation complex [86]. This leads to a decrease in general protein synthesis, but an increase in selective translation of mRNAs coding for proteins that help the cell cope with cellular stress [88].

Many mRNAs with IRES encode proteins that have important roles in cell cycle, proliferation, differentiation and apoptosis. Several proteins that are important regulators in apoptosis such as APAF1 [98], XIAP [99], p53 [100] and c-myc [101] are translated in an IRES dependent manner. During apoptosis, eIF4G as well as other initiation factors and parts of the translation initiation complex such as PABP, are cleaved by caspases [102] which leads to an inhibition of cap-dependent protein synthesis. Even though cap-dependent protein synthesis is targeted in apoptosis induction, protein synthesis can still go on in an IRES-dependent manner. Cleavage products generated from eIF4G and DAP5 can even specifically enhance translation of anti-apoptotic proteins during apoptotsis induction [103,104].

In addition to phosphorylational and caspase cleavage dependent regulation of initiation factors that induce a switch from cap-dependent to IRES-dependent protein synthesis, a switch can also be induced by stalling of elongation [105]. The elongation process consists of recruiting the amino acyl-tRNAs to the A-site of the ribosome, a process involving eEF1A and eEF1B, and to translocate the ribosome relative to the mRNA by one codon, a process involving eEF2 [106,107]. The eEF1Ba subunit of eEF1B mediates GDP/GTP

exchange on eEF1A. The eEF2 is a GTPase and its activity is affected by the stalk structure of the 60S ribosomal subunit, a pentameric complex consisting of ribosomal proteins rpP1, rpP2 and rpP0 [108]. rpP2 has two C-terminal phosphorylation sites, Ser102 and Ser105 [109], and for rpP2 to interact with eEF2, it has to be in the phosphorylated form [110,111]. The eEF2 is regulated by phosphorylation by the eEF2 kinase (eEF2K), and is inactivated upon phosphorylation as the phospho form is unable to bind to the ribosome (for review see [107]). Dephosphorylation of eEF2 is probably mediated by PP2A or a closely related phosphatase [112]. The eEF2K is also regulated by phosphorylation by several kinases such, as e.g. p70S6K, and a phosphorylated form renders the eEF2K less active and hence promotes translation by a decreased eEF2 phosphorylation (for review see [107]).

10% of the cellular mRNA have the potential to be translated by an IRES-dependent mode [113]. So far, IRES-mediated translation is the only cap-independent translational mechanism that has been validated [114-116]. IRES mediated translation provides a way to escape the global decline in protein synthesis that is followed almost by any type of stress and is an energy saving mechanism since it is estimated that 50% of all cellular ATP is consumed in the protein synthesis process. It is also a way to allow selective translation of specific mRNAs, a function that is especially important in regulation of cell death and survival [117]. A switch from global to specific translation allows for selective translation of proteins that are required for cell survival [118,119]. Efficient IRES dependent translation requires cellular proteins known as ITAFs (i.e. hnRNP c1/c2 and hnRNP K [120]) but their absolute requirement seems to be IRES specific [119].

RNA granules

Translational regulation can also be exerted on the level of availability/stability of transcribed mRNA. Cytoplasmic RNA granules serve key functions in controlling the localization, degradation/stability and availability of mRNAs [121], and several distinct types of RNA granules have been identified. After stress induction, mRNA assembles in stress granules where mRNAs are translationally repressed [122]. RNA processing bodies (P-bodies) contain several proteins necessary for mRNA decay and RNA for degradation

or storage accumulates therein [123]. Also other granules, as the IMP1 RNP granules, associates with untranslated mRNA [124].

Protein synthesis in cancer

Cancer cells are more dependent upon protein synthesis than normal cells as they have a higher rate of proliferation. Hence, deregulations of mTOR signaling pathways are considered to play an important role both in cancer initiation and progression [125], and mTOR is aberrantly activated in more than 70% of AML [12]. Deregulation of mTOR can result from activation of upstream effectors of mTOR such as PI3K and Akt and/or over-expression of components in the translational machinery so that they are no longer under the control of mTOR (reviewed in[93]). In addition, several components of the protein synthesis machinery are upregulated cancers [126,127 and refs therein]. As several oncogenes, growth factors and anti-apoptotic proteins have been shown to have an IRES in their 5′-UTR, regulation of their translation escapes cap-dependent regulatory mechanisms and selective translation of these factors contribute to survival of cancer cells under stressful situations and contributes to development and progression of cancer and to apoptosis resistance [128].

Endocytosis and intracellular trafficking

Endocytosis is the process of vesicle formation from the plasma membrane, and in this process, plasma membrane-associated proteins are internalised into membrane-bound transport vesicles. The endocytic pathway begins at the plasmas membrane and ends up in the lysosomes, and along this pathway cargo is either destined for recycling back to the plasma membrane or for degradation. As cargo passes through early and late endosomes, communication with the *trans*-Golgi network and the secretory pathway happens. The secretory pathway functions to deliver secretory and transmembrane proteins and lipids from the ER, via the Golgi apparatus, either to intracellular compartments, to the plasma membrane or to the exterior of the cell. At all stages in this vesicle-mediated transport between the plasma membrane and various intracellular compartments, recognition, tethering and fusion of vesicles are essential for proper destination of the cargo.

Endocytosis supports various cellular functions including nutrient uptake, growth-factor signaling, and membrane homeostasis [129]. Categorically, endocytosis can occur by multiple mechanisms, and can be divided into phagocytosis or "cell eating" of large particles typically restricted to specialized cells such as macrophages and neutrophiles [130] and pinocytosis or "cell drinking", the uptake of fluid and solutes that occurs in all cells. Pinocytosis occurs by at least four basic mechanisms: macro-pinocytosis, caveolae-mediated, clathrin- and caveolin-independent and clathrin-dependent endocytosis [129]. These pathways are thought to vary mechanistically in how the vesicles are formed, which cargo molecules are internalized, how entry is regulated and to what intracellular destination the cargo is delivered.

Clathrin-mediated endocytosis

The best characterized endocytosis pathway is clathrin-mediated endocytosis (CME). It ensures uptake of nutrients and provides a way of transferring and regulating signaling from the exterior to the interior of the cell through surface receptor internalization. CME occurs both constitutively and as a result of ligand binding to surface receptors. Constitutive CME ensures a constant uptake of nutrients, such as e.g. iron, where iron-loaded transferrin binds to the transferrin receptors, TfR [131]. This internalization is not dependent upon ligand binding. On the other hand, cytokine and growth factor receptors are internalized upon ligand binding and endocytosis serves a prominent role in signal transmission from these receptors. While receptor-mediated endocytosis has traditionally been considered an effective mechanism to attenuate ligand-activated responses, more recent studies demonstrate that signaling continues on the endocytic pathway [132]. Protein components of signal transduction cascades can assemble at clathrin coated pits and remain associated with endocytic vesicles following their release from the plasma membrane. Thus, endocytic vesicles can function as a signaling compartment distinct from the plasma membrane.

Several proteins are involved in and essential for the formation of clathrin coated pits and vesicles and for the concentration of transmembrane receptors into these pits. This process and proteins involved are illustrated in Figure 3. Clathrin adaptors, such as adaptor protein 2 (AP2), link clathrin to cytoplasmic determinants of endocytic cargo

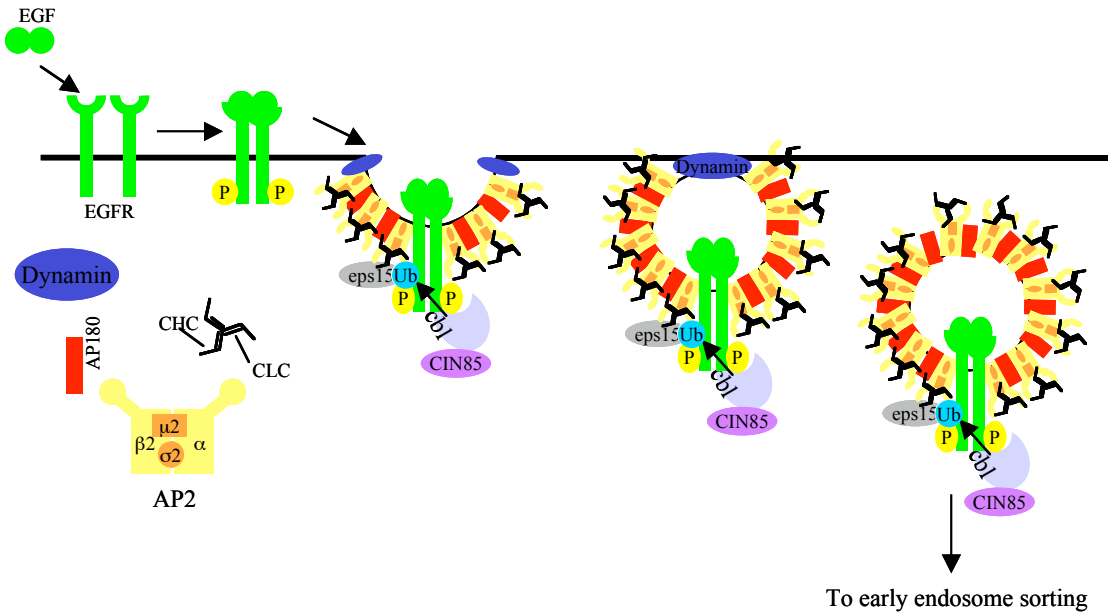


Figure 3. Clathrin-mediated endocytosis of EGFR. The internalization of EGFR is a well described model for ligand-induced endocytosis and is used here to illustrate components that are involved and important for clathrin-mediated endocytosis. The illustration is designed primary to show the function of the proteins that we found modified in anthracycline-induced apoptosis, and will therefore not be complete when it comes to all factors involved in internalization of EGFR. The AP180, AP2 and Eps15 adaptor proteins interact with membrane phopholipids and the intracellular parts of the receptor and recruit the clathrin triskelions to the membrane to assembel a polygonal lattice. The natural curvature of this lattice leads to the formation of invaginated structures known as clathrin coated pits. Phosphorylated EGFR recruits the E3 ligase Cbl which monoubiquitylates EGFR, associates with CIN85 and promotes receptor endocytosis. By the aid of dynamin, endophilins and amphiphysin these pits become a vesicle that is attached to the plasma membrane by a narrow neck. A GTP dependent change in dynamin causes scission and release of the vesicle from the membrane (After [133,134]).

during the formation of clathrin coated pits. The AP2-complex is a heterotetramer and its targeting to the plasma membrane is by the α-subunit. AP180 and Eps15, two other adaptor proteins, bind to AP2 and promotes the growth of clathrin coated vesicles by recruiting the clathrin triskelion. The adaptors also perform scaffolding functions in endocytosis by recruiting accessory or regulatory proteins [135]. The adaptors might serve different functions for endocytosis of different receptors. E.g. phosphorylation of Eps15 is essential for epidermal growth factor receptor internalization, but not for constitutive endocytosis of the TfR, suggesting Eps15 to have supplementary regulatory functions in endocytosis in response to proliferation signals [136].

The clathrin triskelion is a three-legged structure composed of three heavy chains (CHC) and three light chains (CLC). Clathrin will self-assemble into polyhedral lattices under non-physiological conditions [137], but requires adaptor proteins to assemble under physiological conditions [135]. Dynamin is a multidomain GTPase that is recruited to the necks of coated pits where it mediates membrane fission resulting in release of endocytic transport vesicles [138]. After uncoating of the clathrin lattice, the vesicles dock and fuse with early endosomes, a reaction dependent upon Early Endosome Antigen 1 (EEA1) and Rab5 [139]. Acidification of early endosomes leads to dissociation of most receptor-ligand complexes, and ligands destined for degradation traffic within late endosomes to lysosomes, while receptor-enriched vesicles are recycled back to the cell surface [140 141].

Coated pits do not assemble randomly throughout the plasma membrane. Instead, CME is organized at endocytic "hot-spots" that are in part constrained by the actin cytoskeleton [142]. The cytoskeletal network also provides the tracks for the movement of transport vesicles between compartments. Several endocytic accessory proteins with multiple domains for protein-protein and /or protein-lipid interactions, such as Eps15 and intersectin, can function as scaffolding molecules that connect the endocytic machinery to the cytoskeleton [129].

Vesicular recognition and fusion in membrane trafficking

The intracellular membrane trafficking system uses vesicles and tubulovesicular structures to deliver cargo proteins and lipids from one compartment to the next. Several proteins including the Rab GTPases and the SNAREs are involved in recognition, docking and fusion of donor and acceptor compartments to ensure proper transport and delivery of cargo molecules [143].

The Rab family is part of the Ras superfamily of small GTPases and confers recognition, initial docking and tethering of vesicles along the endocytic and secretory pathways [144]. The Rabs display characteristic subcellular localizations, e.g. Rab5 localizes to the early endosomes [145] and to clathrin coated vesicles [146]. The Rabs cycles between an active (GTP-bound) and inactive (GDP-bound) form and associates with a number of effector proteins that determine their specific targeting and function [144].

SNAREs acts downstream of the Rabs, and they confer tight docking and subsequent fusion of membrane bilayers in addition to ensuring specificity and recognition. They comprise three families of membrane associated proteins: The vesicle v-SNARE (synaptobrevin/VAMP), the target membrane t-SNARE (syntaxin) and the additional light chain family (SNAP-25). In addition to the SNAREs, several factors that interact directly with and are required for the ordered assembly of the SNAREs into functional complexes and fusion and trafficking of vesicles, are known. These include among others Munc13, synaptogamin and tomosyn [147].

Endocytosis in cancer

Defects or mutations in proteins related to the endocytic machinery are found in cancer cells. The translocation t(10;11)(p13;q14) found in ALL and AML resulting in the CALM-AP10 fusion protein, is linked to poor prognosis and leukemogenesis [148]. CALM is the non-neuronal homolog of AP180 and involved in clathrin assembly. Exactly how the CALM/AF10 fusion protein contributes to leukemogenesis is not known, but several aspects have been suggested ranging from interference with gene expression [149] to impaired endocytosis resulting in prolonged growth factor signaling and increased proliferation stimuli [148]. Also fusion proteins of Eps15 and endophilin-2 have been identified in AML and ALL [150]. It has been established that impaired downregulation of RTKs is associated with cancer, and mutations or overexpression of proteins that impair receptor internalization and ensure enhanced signaling from growth promoting receptors have been reported. E.g. oncogenic forms of RTKs has been found to lack binding sites for Cbl, the ubiquitin ligase responsible for its internalization [151]. Also Hip1, involved in CME, is overexpressed in cancers [151].

In order to reduce systemic cytotoxicity of anti-cancer drug treatment, the endocytic pathway has been suggested as a specific mean for delivering therapeutic drugs to cancer cells. The TfR has been explored as a target for delivering therapeutics to cancer cells due to its increased expression in malignant cells, accessibility on the cell surface and constitutive endocytosis [152]. Also the LDL-receptor family and folate receptors have been suggested as mediators of cellular drug uptake [153,154].

Aims of the study

We initiated a study of the molecular mechanism of action of the anthracycline class of chemotherapeutic drugs to:

1: Learn how these drugs initiate and execute apoptosis in AML and identify proteins/pathways affected by the anthracyclines (curiosity-driven research).

2: Based on above, hopefully pinpoint proteins/pathways that might become targets for rationally designed drugs.

3: Learn about the shortcomings of anthracyclines in apoptosis-induction and propose suitable drugs for combination therapy to overcome some of the weak points of these drugs.

Summary of Papers

Summary Paper I

By 2DE analysis the p23 co-chaperone of Hsp90 was identified as a caspase target in cell death induced by atnthracyclines (and various other death-inducing agents). Both effector caspases 3 and 7 could cleave p23, which occurred at two sites (D142 and D146) in the C-terminal tail of p23. The cleavages did not induce dissociation of p23 from the Hsp90-complex. The presence of the Hsp90 inhibitor geldanamycin (GA) enhanced p23 cleavage both *in vitro* by recombinant caspases and in intact cells exposed to anthracyclines and GA. GA also increased the apoptotic effect of the anthracycline. We postulate that the cleavage of p23 could compromise its ability to chaperone client proteins in the Hsp90 multi-protein complex and hence contribute to apoptosis induction.

Summary Paper II

We show that anthracyclines target the protein synthesis machinery by modulating key proteins involved in protein synthesis. The result is a switch from a regular cap-dependent to non-cap-dependent synthesis. This switch is due to truncation, degradation and altered phosphorylation status of proteins controlling cap-dependent initiation, polypeptide elongation and specific mRNA translation. Targeting the protein synthesis machinery did not shut down synthesis all together, but resulted in a translational upregulation of proteins with pro-survival functions such as ER chaperones. Co-administration of the anthracycline DNR and protein synthesis inhibitors (added in the pre-apoptotic phase) enhanced apoptosis both in AML cell lines *in vitro* and in several animal models of AML. This death-enhancing effect of the protein synthesis inhibitor could be due to disruption of the anthracycline-induced, presumably IRES-dependent synthesis of survival proteins. We postulate that the switching on of IRES-dependent synthesis of survival proteins is an “Achilles heel” in anthracycline action and that co-

administering anthracyclines with a protein synthesis inhibitor in the pre-apoptotic period should be considered for developing improved anthracycline-based AML therapy.

Summary Paper III

Both by conventional 2DE analysis and an immunoblot screen we found that clathrin-mediated endocytosis was targeted in anthracycline-induced cell death. Several proteins important for clathrin-mediated endocytosis were subjected to caspase-or proteasome-mediated cleavage or degradation. This was accompanied by compromised uptake of transferrin and increased surface accumulation of the transferrin receptor. Clathrin light chain (CLC) was the first of the proteins involved in clathrin-mediated endocytosis to be targeted, and was completely degraded (presumably by the proteasome) before the onset of morphological signs of apoptosis and before detectable caspase activation. The degradation of CLC was also detected in blasts isolated from patients receiving induction therapy. Inhibition of the drug-induced CLC degradation resulted in reduced shutdown of transferrin endocytosis and reduced accumulation of surface receptors indicating that CLC plays an important role in clathrin-mediated endocytosis. Also receptors important in life-and-death signaling such as Fas/CD95 and IFNγ receptor accumulated on the surface in response to drug treatment, and co-treatment with monoclonal Fas antibody and IFNγ enhanced anthracycline-induced apoptosis. The anti-apoptotic phosphorylational activation of STAT5 induced by IFNγ was abrogated upon drug-treatment. This indicates that accumulation of surface receptors due to targeting of proteins important in clathrin-mediated endocytosis can play a role in induction or execution of anthracycline apoptosis.

Methodological considerations and aspects

AML cell lines

Establishment of stable leukemia cell lines bearing gene rearrangements and secondary gene mutations characteristic of different AMLs have for decades been utilized for *in vitro* studies of aspects governing AML development and drug response. Cell lines are tools that can be exploited with some benefits, but the artificial aspects of cellular models based on selected, immortalized tumor cells should not be neglected. Therefore, to prove a principle/mechanism to be clinically and generally relevant for AML, one should address several representative cell lines and also include patient material in the study. In this study we have used the following AML cell lines, in addition to available patient material, to study the mechanisms of anthracycline action:

NB4: The NB4 cell line (FAB-M3) was originally isolated from bone marrow cells from an APL patient in relapse [155]. The cell line bears the t(15;17) translocation and differentiates into granulocytes upon ATRA-treatment [156]. Upon treatment with arsenic trioxide and drugs such as anthracyclines and the Hsp90 inhibitor 17-AAG, the NB4 cells undergo apoptosis [157-159]. The features of the cell line are extensively reviewed in [160].

HL60: The HL60 cell line (FAB-M2) is a non-APL cell line, but exhibits an APL phenotype when it comes to ATRA-response. The cell line was initially derived from a patient with APL, however represents a less differentiated/more immature form of APL than the NB4 cell line, lacks the t(15;17) translocation and can be differentiated into a number of different cell types along the myeloid linage [161]. The cell line has been extensively used to study AML proliferation, differentiation, oncogene expression and apoptosis induction.

IPC-81: The IPC-81 cell line is an APL cell line derived from the Brown Norwegian rat acute myelocytic leukemia (BNML) [162]. It has been widely studied as a model system for AML apoptosis-induction both by anthracyclines, phosphatase inhibitors and cAMP [163] [164].

Animal models of AML

Even though cell lines are useful models for *in vitro* studies of cancer development and treatment response, they do not precisely recapitulate the *in vivo* settings of human AML. For this reason, AML animal models have been developed in which AML treatment modalities can be tested and studied. In this study we used several transplantable AML models to test a combination treatment consisting of an anthracycline and a protein synthesis inhibitor that had proved successful in cellular *in vitro* studies.

The BNML rats are considered a good rat model for human AML as it closely resembles human AML with respect to growth characteristics and response to chemotherapy [165,166]. The BNML rat model has been employed in preclinical evaluation of several treatment modalities and has been used to explore the action of and resistance against cytostatics like cyclophosphamide [167], 5-fluorouracil [168], acetyldinaline [169] and inhibitors of angiogenesis [170]. This is a good model as it is syngenic. Hence, the immune system of the model is intact and the effect the immune cells exert on the cancer cells during therapy will be implicated in the therapy readout.

To supplement the BNML rat model, we also used nude immuno-incompetent *rnu/rnu* Rowett rats as receivers of IPC-81 cells [171].

The nonobese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) exhibit multiple defects in both adaptive and innate immune response [172] and is a successful model for transplanted human AML with high engraftment rates [173,174]. In this study, we used the beta2 microglobulin knockout NOD/SCID $B2m^{null}$ mice as receivers of NB4 cells. This strain is an excellent receiver of human leukemic AML cells as the innate immunity are further reduced due to lack of natural killer cells [175].

To induce leukemia, cells were injected into the tail vein. Establishment of leukemia with homing and engraftment in the spinal cord, sternum and femur are shown in Figure 4.

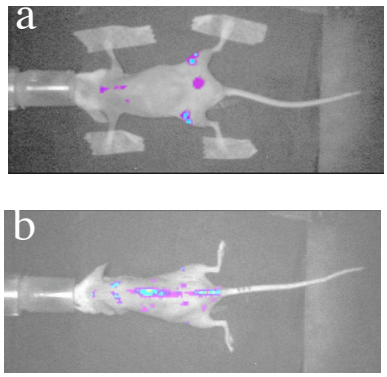


Figure 4. Engraftment of GFP-positive IPC-81 cells in BN/Rij rat. Development of leukemia was followed and imaged in an eXplore Optix time-domain imager (ART Advanced Research Technologies Inc., Montreal, CA). (a) Ventral side of rat showing GFP-signals in the femur and sternum. The signal from the in the bladder is unspecific. (b) Dorsal side of rat showing GFP-signal from the spinal cord. The rats were imaged 3 weeks after injection of cells (picture taken by I. Brønstad).

Anthracycline concentrations and clinical relevance

Several studies have pointed to different mechanisms of action of the anthracyclines (see Introduction for details). An important notice in this context is that the mechanism of cytotoxicity of the anthracyclines varies depending on the drug concentration used. To study the mechanism of drug action that is relevant for the clinical cytotoxicity of these drugs, it its therefore important to use concentrations that are achieved or sustained in patients receiving therapy. Findings by Muller et al. illustrate this point. They report that at pharmacological relevant doses of doxorubicin, the main killing mechanism against leukemic cells is induction of apoptosis and not oxidative DNA damage [176]. Also, DNA breakage due to reactivity of ROS is probably only relevant at supra-clinical doses, at clinically relevant doses, the generation of DNA breaks is probably due to the inhibition of topoisomerase II [24]. The different effects of anthracyclines at high and low doses are also evident in their ability to induce an apoptotic cell morphology. We found that low doses of DNR in IPC-81 cells induced apoptosis with classic morphological features such as membrane blebbing and chromatin condensation. However, at elevated doses, cell death with no classical signs of apoptosis occurred (Paper II). We called this “frozen “ cell death. Upon removal of the drugs by washing, the cells displayed classic apoptotic features. This phenomenon has also been reported for other leukemic cells and cell lines at elevated drug doses [59,177,178].

For a study like this to have clinical relevance, it is therefore essential to mimic clinically relevant concentrations of DNR in experimental set-ups. However, finding the right drug concentrations to use is not straightforward. The peak plasma concentrations of anthracyclines like DNR are estimated to be in the range from 0.3 to 5 μM, and most often between 1–2 μM [28]. However, extracellular drug concentration does not necessarily directly mimic the intracellular drug concentration as different cell lines show differences when it comes to drug accumulation and ability to accumulate the drug in intracellular compartments such as the lysosome which will decrease drug cytotoxicity [179,180].

In our cell culture studies we used doses of DNR between 10 nM and 8 μM, i.e. most of our experiments lie within clinically relevant doses [28]. When doses of DNR were used that exceeded the reported plasma peak concentration of 5 uM, this was to ensure rapid and complete effects, and also to counter the argument that what we observe was a response only relevant at sub-apoptotic concentrations of DNR and only reflect general cell stress and irrelevant as soon as cells start to undergo apoptosis. Major findings obtained with elevated doses were always reproduced with lower, clinically relevant doses to confirm the relevance of the findings. This accounts for e.g. the synergistic effect of DNR and CHX and the DNR-induced dephosphorylation of mTOR substrates (Paper II). IDA is a more lipophilic drug than DNR, accumulates more easily in cells and is considered about 4-8 times as efficient as DNR when it comes to apoptosis induction [181]. We therefore used IDA concentration ranging between 100 and 720 nM. Even though it is not straightforward to determine the proper drug concentrations to use or foresee the actual intracellular concentration of the drugs, that several of our drug-induced findings such as rpP2 dephosphorylation (Paper II), CLC degradation, transferrin endocytosis decrease and Fas-R surface accumulation (Paper II) were also detected in blasts isolated from patients receiving chemotherapy, convince us that our findings are clinically relevant even though we also obtained some of these results by using doses that are higher than the ones achieved in the patients during therapy.

In our animal studies on the effect of co-treatment of anthracyclines and CHX, we also used clinically relevant drug doses. Typical treatment concentrations of DNR (Cerubidin, Aventis) for i.v. infusion are in the range 0.5 – 3 mg/kg per infusion (Felleskatalogen,

2007, 49. utgave. Felleskatalogen AS, Oslo). We used 0.5 or 1.5 mg/kg in animals on three consecutive days. Of interest, we found a significant prolongation of survival with administration of 1.5 mg/kg DNR, which was further enhanced by CHX, but with the lower dose of 0.5 mg/kg of DNR, we found little effect of DNR alone, but a stronger synergism with CHX (Paper II). The IDA doses we used are somewhat higher on mg/kg than the common clinical dose (12 mg/m^2) used for human AML. We suspect the need for the relatively high doses of IDA to be due to pharmacokinetic differences, presumably suboptimal absorption of IDA from the intragastric tube used to administer that drug.

While we are quite confident that the CHX concentrations used in cell culture experiments inhibited protein synthesis during the entire experiment, we did not measure the effect of CHX on protein synthesis in the AML cells in our *in vivo* models to establish that CHX for certain inhibited protein synthesis at the doses administered (mice: 5 mg/kg).This was because doses between 2-5 mg/kg have been reported to be sufficient to inhibit protein synthesis in mice [37]. In rats, the doses we used were lower, 0.8-1.5 mg/kg. The reason for this was that the rats were more sensitive to CHX treatment and were killed by higher doses of CHX.

Proteome analysis: 2DE and COFRADIC

The response of a certain drug on gene expression has for the last decade been a popular way of studying the effects of chemotherapeutics. The development of microarray techniques has provided an enormous amount of information on how drugs effect the expression of certain genes. However, drug-induced changes in gene expression have not always been reflected at the protein expression level. Even though the literature on how anthracyclines regulates gene expression is not that big and that further such studies are warranted, we decided to choose a proteomic approach in order to get direct information about the effects on cellular proteins. Another important reason for choosing a proteomic approach over a genomic approach is that we can detect drug-induced changes in post-translational modifications, such as phosphorylation, which is an important cellular regulatory mechanism when it comes to transducing signals that are related to apoptosis [182].

Studying how the proteome of a cell is affected by a drug provides information about the cellular targets/proteins that are important for the mechanisms of action of the drug and about which cellular signaling pathways that are activated in response to the drug. Two-dimensional polyacrylamide gel electrophoresis (2DE) together with mass spectrometry (MS) have successfully been used to identify changes in the proteome linked to apoptosis induction or execution [183-190]. Although 2DE because it also reveals posttranslational modifications such as changes in the phosphorylation status [191], the limitations of the 2DE technique in addition to being time consuming and problematic when it comes to reproducibility, is its failure in detecting low copy number proteins, hydrophobic proteins and proteins that are either very small or large or highly basic. For these reasons, we realized the need to study the proteome of apoptotic cells also by additional methods than 2DE.

To get a "global" view of protein changes associated with DNR-induced apoptosis, we used the non-gel high throughput technique of combined fractional diagonal chromatography (COFRADIC) for sorting of protein amino-terminal peptides [192-194]. The technique is developed by K. Gevaert and J. Vandekerckhove at the Department of Medical Research and Biochemistry, Ghent University, Belgium. The peptide isolation procedure, MS/MS and data analysis that are part of this study, were preformed by Petra Van Damme in the lab of K. Gevaert (COFRADIC study on NB4 cells, Paper II) or by Anne Døskeland in the lab of K. Gevaert and at the Probe Unit, University of Bergen (COFRADIC study on IPC-81 cells, Supplementary Table 3).

COFRADIC uses enforced changes in the chromoatographic qualities of a subset of peptides to reduce the complexity of the protein sample. In our study only the subset of N-terminal peptides in a trypsin-cleaved lysate were isolated and analyzed by MS/MS [193,194]. Since each protein only has one N-terminus, each protein is therefore represented only by one peptide. By differential labeling of peptides either chemically with oxygen-16/18, or metabolically with SILAC-labeling, relative changes in protein expression between two different samples can be measured by MS. By this method data about new protein N-terminals that are generated as a result of protease-mediated activities are achieved, and the exact cleavage site is identified. In addition, by comparing the ratio of

N-terminal peptides, information about proteins that are downregulated (e.g. due to proteasomal activity) are also obtained. The COFRADIC method for sorting and analysis of peptides can also be used on other types of peptides in addition to the N-terminal ones, such as methionyl, cysteinyl and phosphorylated peptides.

The diagonal peptide chromatography consists of two consecutive, identical peptide separations with an enzymatic or chemical alteration of the side-chain structure of the selected peptides in between the two separations. These selected and altered peptides then acquire different chromatographic properties and can thereby be segregated from non-altered peptides in the secondary peptide separation. The procedure for sorting of N-terminal peptides is outlined in Figure 5.

In our first COFRADIC study (NB4 cells, Paper II) differentially Oxygen-16/18 labeling was used. O-16/18 labeling has the advantage that it is done post-metabolically in the reaction catalyzed by trypsin and therefore applicable on every proteolytic digest independent of its origin of sampling; being tissue extractions, body fluids or cell culture lysates. However, O-16/18 labeling was in the second COFRADIC study (IPC-81 cells, Supplementary Table 2) replaced by SILAC labeling. SILAC labeling has some advantages over O-16/18 labeling. O-16/18 labeling is not totally stable and back-exchange can occur in acidic environments. Also, spacing, between the light and heavy isotopes is only 4 Da, making the determination of abundance ratios not so straightforward as with SILAC labeling where 13C/12C-arginine is used and peaks more easily declustered in the MS-analysis as the spacing is increased to 6 Da. However, metabolic SILAC labeling of cells has to be done in medium with dialyzed serum and for some cell lines this could affect viability and response to the experimental treatment. Also the cellular 13C6-arginine to 13C5-proline conversion during SILAC labeling is a challenge that will complicate MS analysis. To avoid this, the concentration of arginine in the medium was reduced and the concentration of proline was slightly increased.

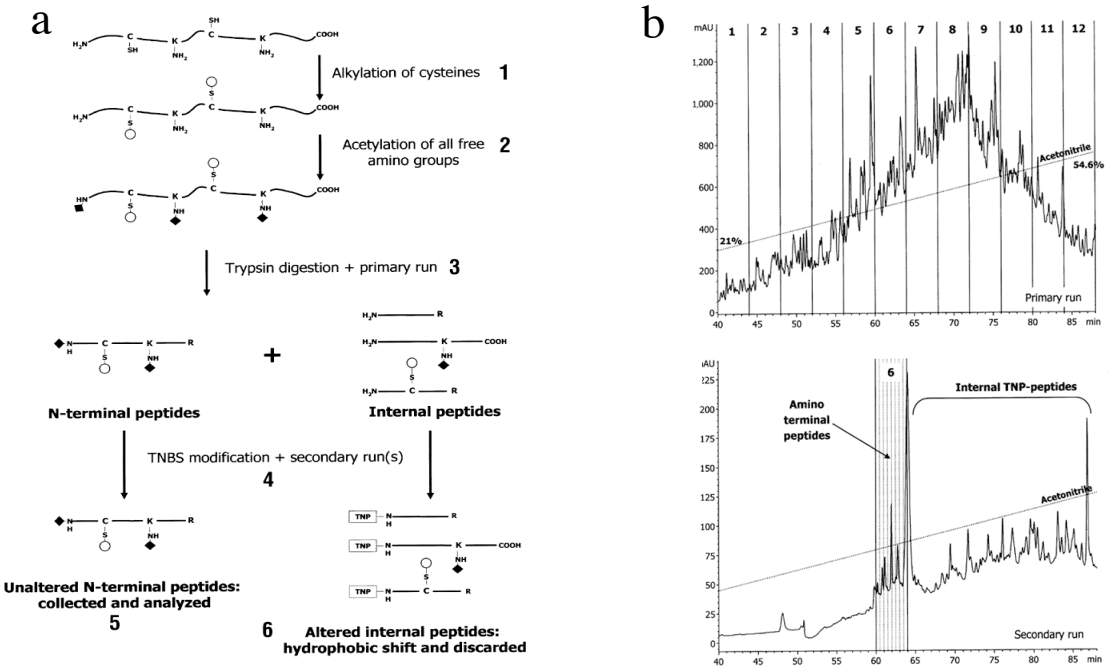


Figure 5. Scheme summarizing the chemistry and chromatographic steps during N-terminal peptide sorting utilized by COFRADIC. (a) (1) Protein-cystein residues are first alkylated using iodoacetamide (open circles). (2) Then all free amines (α -and ε-amines) are acetylated (filled diamonds), and the proteins are digested with trypsin (3) that will only cleave C-terminal to arginine residues. This creates two types of peptides: N-terminal peptides with a blocked N-terminus and internal peptides with a free N-terminus. After the primary RP-HPLC fractionation of the generated peptide mixture, all peptides present in one HPLC fraction are treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (4). Only the internal peptides with an unblocked N terminus are altered to trinitrophenyl-peptides (TNP open box). This reaction will induce a strong hydrophobic shift in the peptides and these peptides will shift out of their original position during the secondary chromatographic run that is identical to the first RP-HPLC separation. The N-terminal peptides are unaltered by the TNBS reaction and will thus elute at exactly the same time interval as during the first run and can be collected and analyzed further by LC-MS/MS (5). The internal peptides labeled with TNBS are discarded from further analysis (6). (b) Peptide elution profiles during the primary and secondary run of N-terminal peptide sorting. During the primary run of chromatographic fractionation of the tryptic protein digest, 12 fractions are collected and treated with TNBS to block the free amines. When the TNBS-treated fractions are separately rerun on the same column and under identical conditions, the internal trinitrophenyl peptides shift to later elution times (hydrophbic shift) while the unaltered N-terminal peptides elute within the same time interval and can be collected in a number of secondary fractions and analyzed by LC-MS/MS. Adapted from [192].

Quite puzzlingly, when analyzing COFRADIC data, we detected unexplainable cleavages in our vehicle treated cells (Ctr) that were not normally seen in similar experiments done with Jurkat T cells (personal communication by P Van Damme and A Døskeland). That is, also internal N-terminal peptides were detected in the Ctr-sample (which theoretically should only contain one peptide per protein, the real N-terminus). The APL cell lines

(such as NB4 and IPC-81) are known to have large amounts of granules containing proteases [195]. The most likely explanation for the presence of internal peptides in the Ctr-sample is that these proteases become activated upon sample preparation and are not inhibited by the cocktail of protease inhibitors present. However, the ratio between identical internal peptides generated in the Ctr-sample and the anthracycline-treated-sample, were close to one, so these peptides were considered background noise in the system and subtracted from the analysis, and therefore considered not to influence the result of the study.

Even though COFRADIC is a sensitive high throughput method, it has its limitations. Some caspase cleavages will not be detected. This is the case if the caspase generated C-terminal peptide does not contain an arginine for trypsin cleavage. For this reason, we were not able to confirm the cleavage of p23 by the COFRADIC since the caspase cleavage creates a C-terminal fragment that does not contain a trypsin cleavage. A newly generated fragment could also be lost in the MS/MS-analysis if it is to long.

Another surprising notion was that none of the endocytosis related proteins detected by an immunoblot screen to be modified in anthracycline-treated cells (Paper III), were identified as caspase targets or as being downregulated in any the two COFRADIC studies that were conducted. First, this indicates that even though COFRADIC is very sensitive when it comes to detection limits, it does not pick up all proteins and proteins can be lost during sample preparation, chromatographic analysis or MS analysis. The lack of a trypsin cleavage site in the generated C-terminal peptide can explain the missed detection for some of these proteins. A second plausible reason why endocytic proteins are under represented in the data obtained from these studies, is that they are not highly expressed in the cell or not properly dissolved from membranes during sample preparation.

In the first COFRADIC study conducted on NB4 cells, we found that the majority of newly generated N-terminal fragments were due to caspase mediated cleavage and only a small fraction was due to cleavage by other proteases. However, in the second study conducted on IPC-81 cells in the presence of IDA and the proteasome inhibitor bortezomib, among the proteins we report in this study (which are only the ones that are

related to intracellular trafficking) only one cleavage was ascribed to correspond to a caspase cleavage site, the rest of the cleavages were due to the action of other proteases. This is quite puzzling, as anthracyclines are known to activate effector caspases [196]. The reason for this is not obvious, but it is possible that the proteasome inhibitor also inhibits certain caspases. This is plausible since the proteasome possesses proteolytic activity that resembles the caspases (e.g the cleavage of AP2-α reported in Paper III and [197,198]. A second plausible explanation for this observation is that IAPs, degraded by the proteasome upon apoptotic stimuli [199] are stabilized in the presence of bortezomib and serve to inhibit caspase activity.

In the COFRADIC studies performed, we did not treat the cells with anthracyclines alone. The first study was performed in the presence of CHX together with DNR (Paper II). The reason for this was to ensure that the changes we identified were not due to newly synthesized proteins, as we wanted to identify modifications in proteins already present in the cell. Having established that CHX merely serves to enhance the effect of DNR and not change its character per se, we consider the changes obtained in this study to represent effects caused by DNR and not CHX. In the second study, IDA was supplemented with the proteasome inhibitor bortezomib. This was done both to inhibit further proteasomal degradation of protease-generated peptides [200], but also because we wanted to study the combined effect of IDA and botezomib since this combination is successful in inducing apoptosis in cancer cells [201]. While the presence of bortezomib could influence the activity of IDA to a stronger extent than CHX would, COFRADIC results from IPC-81 cells treated with IDA alone, shows that the cleavage pattern is quite similar in the two samples (personal communication A. Døskeland) providing evidence that the protein targets reported in Supplementary Table 2 are most likely due to the action of IDA.

Immunoblotting versus COFRADIC; supplementary methods

A large part of the data obtained in this study is achieved by the use of proteomic methodology. This is a suitable approach for an open-end study such as the one we have undertaken here and produces unpredictable and exciting results. Nevertheless, even though proteomics can guide us in new directions, it is a technique that has to be

supplemented by other methods for verification. Firstly, this is because even though a proteomic study is considered a "global" study of changes in the proteome, far from every change will be detected (see above). Hence, one cannot be sure that the results achieved represent the full story. A proteomic study should therefore only be used to get an indication about what pathways one should look deeper into. Secondly, a proteomic study does not necessary indicate the nature of the protein modification. Therefore, hits should be verified by e.g. immunoblotting. Given that the appropriate anitbodies against suitable epitopes are used, immunoblotting will give information about whether a protein is totally degraded or whether a stable fragment is produced. Thirdly, data obtained by COFRADIC does not always indicate to what extent a protein is cleaved. Due to limitations in manpower and time, we have so far only verified a few of the proteins found by COFRADIC to be modified. We realize that several of the major findings should be verified by immunoblotting both to determine the completeness of their degradation/cleavage and to get information about the nature of the modification and whether a stable cleavage product is produced. This is important since caspase generated cleavage products are not necessary inactive, but can have distinct functions compared to the parent protein [202,203]. A hit by COFRADIC should therefore not automatically be interpreted as a loss of function event, but should be further investigated.

In vitro protein synthesis

In this study we used *in vitro* protein synthesis translation by the Rabbit Reticulocyte Lysate (RRL) to study the effect of DNR on mRNA levels. The RRL-system is an efficient *in vitro* eukaryotic protein synthesis system widely used for translation of RNA, but the method clearly has its limitations. Even though translation of proteins in RRL is thought to be quite representative, e.g. high molecular proteins are often poorly translated due to their large size. For this reason, the value for PDI (spot 2, Figure 4 and Table 1, Paper II) is not given as an accurate value, but rather estimated due to the fact that PDI was not present in every gel we used for calculation. The reason why a calculated value for cutA (spot 4, Figure 4 and Table 1, Paper II) is not shown, is that the observed pI-value for cutA does not correspond with the calculated one (pI 4.45 versus 5.15 respectively), hence we were not able to detect cutA on the gels with *in vitro* translated

proteins. cutA is probably subjected to some kind of posttranslational modification in cells that is not mimicked in the *in vitro* translation system.

From our *in vitro* translation experiments, we noted that translation of rpP2 (spot 1, Figure 4 and Table 1, Paper II) was surprisingly low compared to other proteins with similar molecular weight. This could reflect that there is very little rpP2 mRNA in the cells or it could reflect that translation from rpP2 mRNA is highly regulated. rpP2 is a ribosomal protein and has a 17 nucleotides long 5`TOP in its 5´-UTR (Figure 6). RRL has been shown to be absent or limiting in certain factors required for efficient translation, and supplementing RRL with these can enhance translation of proteins with a 5`TOP [204,205]. While the efficient translation of proteins with 5`TOP was initially thought to depend on phosphorylation of ribosomal S6 by the p70S6 kinase, this has later been questioned [94]. We did not study the phosphorylation status of S6, but we found that DNR resulted in dephosphorylation and cleavage of p70S6K, probably leading to decreased S6 phosphorylation. The enhanced synthesis of rpP2 in cells treated with DNR therefore supports the view that translational regulation of mRNAs with a 5`TOP is independent upon S6. Another ribosomal protein translated with much better efficiency in our system is eEF1Ba (spot 6, Figure 4 B,C, Table 1, Paper II). This protein has a 5`TOP consisting of 10 pyrimidines, probably making its mRNA less dependent upon specific translation factors (Figure 6).

rpP2

5´-ggtttaaccc cgcctcttgc gtcggcg**cct tccttttcct ccct**gtcgcc accgaggtcg cacgcgtgag acttctccgc cgcctccgcc gcagacgccg ccgcgatg-3`

eEF1Ba

5`-cgcg**ctctct ttct**gctgct ccccagctct cggatacagc cgacaccatg-3`

Figure 6. The 5`-UTR of rpP2 and EF1Ba. The inititation codon is underlined. The oligopyrimidine tract is in **bold.**

Flow cytometric (FACS) analysis

In this study, as a supplement to microscopy, flow cytometry was used to determine apoptosis, either by AnnexinV and propidium iodide (PI) staining or by forward and side scattering qualities. AnnexinV detects externalized phosphatidylserine (PS). PS externalization is regarded an early event in apoptosis induction as PI is an "eat me" signal used for recognition of apoptotic cells and bodies for clearance [206]. Forward (size) and side scattering (granularity) measures qualities regarded as being modified later in apoptosis than e.g. PS externalization.

FACS-analysis was also used to measure transferrin endocytosis and accumulation of surface receptors. The strong fluorescence of the anthracyclines made these experiments a challenge. IDA is less fluorescent than DNR and was therefore used for apoptosis induction in experiments involving FACS-analysis. The Argon Laser (488 nm) excites IDA, which then gives a broad emission spectra. We therefore used an alternative red laser (635 nm) that does not excite IDA. For detecting surface molecules with commercially unconjugated antibodies or antibodies conjugated to FITC, we used a secondary antibody conjugated for readout by the red laser. To measure internalized transferrin, we used both transferrin conjugated to Alexa488 or Alexa647. The internalized transferrin conjugated to Alexa488 gives ten times stronger signal than IDA in the FL-1 channel (515 nm-545 nm), therefore the Alexa488 was suitable to use even in the presence of an anthracycline.

For determination of surface receptors, extracellular flow cytometry was used. We did not combine this with analysis of the intracellular pool of the receptors. Doing so would provide information about the amount of receptors present on the surface relative to the intracellular pool.

Discussion

This study was undertaken to learn more about how the anthracycline class of drugs, used successfully for several decades in the treatment of cancers, induce apoptosis. By studying their mechanism of action, we can learn why they so efficiently kill cancer cells and identify specific proteins that can serve as targets for intelligent drug design. We can also discover the weaknesses, the "Akilles heel" of the drugs, and thereby be able to increase the efficiency of the anthracyclines by combination therapy.

Even though much is known about the mechanism of anthracycline action, the results obtained in this study revealed that there is still much to learn. The majority of the targets we identified are probably not anthracycline-specific, but are part of a common downstream cascade of apoptotic events representative for several apoptosis-inducing drugs. This accounts for several of the capsase cleaved proteins, such as e.g p23. On the other side, the targeting of cap-dependent translation through dephosphorylation of mTOR substrates could be a more anthracycline-specific effect and not a common event of all apoptosis inducing agents. Targeting of cap-dependent protein synthesis has previously been reported for cells exposed to stress [207], but it does not seem to be a common drug targeting pathway as we failed to detect similar changes in AraC-induced apoptosis and in apoptosis induced by drugs isolated from marine micro-organisms (Gausdal, Herfindal, Døskeland, unpublished results).

The success of the anthracyclines is most likely due to the fact that they do not target one pathway or one specific oncogene, but affect a number of pathways important for cellular survival and cancer progression. Nevertheless, additional targeting of these pathways/proteins by specific drugs enhances the apoptotic-inducing effect of the anthracyclines. That this is true both for GA, Fas ligandation and proteasome inhibitors (anthracyclines also target several of the proteasome subunits and bortezomid enhance anthracycline-induced apoptosis, unpublished results) have been shown both in this study and by others. This shows that even though the anthracyclines are successful, enhancing their efficiency by combinational therapy is possible. The combinational therapy can either serve to further target the same pathways as the anthracyclines or specifically

target pathways that the anthracyclines fail to inhibit or survival pathways that they induce. A combination with the Hsp90 inhibitor GA is already considered for use in AML (personal communication BT. Gjertsen), and GA is in clinical trial for several other cancers [20]. Proteasome inhibitors are also postulated for treatment in AML [208]. In addition, we have shown that administration of anthracyclines together with drugs that inhibit stress-induced survival protein synthesis could be a promising therapy strategy.

When studying the interplay between a cell and an apoptosis-inducing drug, it is not always straightforward to discriminate whether the observed effects are the consequences of direct drug-mediated actions or rather reflects the cellular response to the drug. It is also not always obvious whether a specific cellular response will contribute to apoptosis or fight it. The drug-induced synthesis of survival proteins that we report in Paper II has been interpreted as a cellular response to fight apoptosis. However, calreticulin, one of the proteins preferentially synthesized during this period, are pre-apoptotically exposed on the surface of cells to ensure the engulfment of apoptotic tumor cells by specialized cells of the immunesystem [209]. Hence, induction of immunogenic cancer cell death allowing the immunesystem to contribute to eradication of dying tumor cells is triggered by the exposure of calreticulin. Therefore, maybe the reason for increased synthesis of calreticulin is not primarily to withstand cellular stress and hence play an anti-apoptotic effect, but rather to ensure recognition of pre-apoptotic cancer cells by cells of the immunesystem and ensure clearance of apoptotic cells in an organism.

In this work we report the shutdown of clathrin-mediated endocytosis (Paper III) and a decreased overall protein synthesis coupled with an increase in the synthesis of a specific subset of proteins (Paper II). Both endocytosis and protein synthesis are cellular pathways that are highly energy demanding. Whether a cell shall commit suicide through the apoptotic pathway or rather pursue down the necrotic pathway is often a question of energy supply [210]. Apoptosis is an energy demanding process that can result in necrotic cell death if the energy supply is limiting. Necrotic cell death triggers an unwanted inflammatory response that is stressful to the organism. From an evolutionary point of view, a cellular response that would ensure apoptosis rather than necrosis would therefore be favored. By shutting down highly energy demanding processes, the cell ensures the

presence of enough ATP to commit to an apoptotic rather than necrotic cell death, a response favorable for the organism. While autophagy is a way of "self-eating" to provide nutrients and energy to stressed cells [211], we did not see clear evidence of induction of autophagic cell death in DNR-treated cells. This could be due to near complete downregulation of the eEF2K that is involved in autophagic cell death [212]. Since the cell does not have the opportunity to sustain energy uptake through autophagy in anthracycline-induced cell death, it might respond to the drug to shut down processes that are highly energy demanding in order to ensure an apoptotic route to cell death.

Anthracyclines and Fas/CD95

A majority of chemotherapeutic agents used in clinical oncology are effective against cancer cells because they induce apoptosis. Although they trigger apoptosis through several mechanisms and despite the variance in chemotherapeutic initiation processes, the release of cytochrome c from mitochondria followed by activation of effector caspases such as caspased-3 and -7 [213] are believed to be a final common pathway in chemotherapy-induced cell death [30,214]. Chemotherapeutic drugs can also induce the upregulation of death ligands or their receptors, and induction and/or execution of apoptosis may therefore be dependent or independent of death receptor signaling. The involvement of the Fas signaling pathway in drug-induced apoptosis has been most extensively studied. Fas-mediated and chemotherapy-induced apoptosis can converge at the level of the receptor, DISC formation, activation of the initiator caspase-8, at the level of the mitochondria, or at the level of downstream effector caspase activation [215]. However, there is controversy regarding the involvement of the Fas pathway in the apoptotic response of tumor cells to anti-cancer drugs. While anthracyclines have been shown to be independent upon activation of Fas-R death signaling to induced apoptosis [39,40], anthracyclines can still kill through activation of Fas [33]. In addition, the activation of Fas-R did enhance anthracycline-induced cell death [216,217]. However, cells selected for resistance towards Fas-agonists failed to show cross-resistance to anthracyclines [218]. We have shown that anthracyclines induced a protein synthesis independent accumulation of Fas-R on the cell surface and that treatment with monoclonal Fas antibodies enhanced anthracycline-induced apoptosis (Paper III). However, others have reported that treatment

with anthracyclines did not increase the surface expression of Fas-R [219]. The reason for this is not clear, but it can be due to the fact that they used much lower doses than we did and that proteasome/caspase activity resulting in compromised clathrin-mediated endocytosis or triggering of exocytosis of the cytoplasmic pool of Fas-R [220] was not activated at the studied time point. That activation of Fas-R enhanced anthracycline-induced cell death despite reduced receptor internalization, supports the findings of Austin et al. that receptor internalization is not a requirement for signaling [221]. However, reports exists stating that internalization is necessary for death signaling [222], so our results could also reflecte that internalization is not completely compromised despite accumulation of the Fas-R and a reduced transferrin uptake.

After the dawn of the "proteomics era", Fas-induced cell death has been quite extensively studied [183,193,223,224]. In addition to being similar when it comes to the death enhancing effect of co-treatment with protein synthesis inhibitors, our main impression is that anthracycline- and Fas-induced apoptosis are also similar when it comes to downstream caspase targets (Paper II and [183,193,223,224]). An intriguing finding that links the two pathways and that can help explain the similarity of Fas-induced cell death and anthracycline-induced cell death is the cleavage of nascent polypeptide-associated complex, NAC (Paper II, and Supplementary Figure 1). NAC has been found in complex with FADD, is thought to modulate FADD-mediated signaling [225] and is cleaved in Fas-mediated cell death [223]. FADD is a critical mediator of signal transduction pathways activated by members of the TNF-receptor superfamily such as the Fas-receptor [226]. Since the expression of NAC modulates FADD-mediated signaling, one could speculate that anthracycline-induced cleavage of NAC will disrupt the NAC/FADD complex and liberate FADD to be free to mediate Fas-like death signaling.

Discussion Paper I

In this work we report that p23 is cleaved in DNR-induced apoptosis and speculate that cleavage of the C-terminal tail of p23 could play a role in induction/execution of apoptosis. This was based on the fact that the C-terminal tail is important for p23´s chaperone activity [227,228], and we hypothesized that the cleavage could compromise both

the autonomous chaperone activity of p23 and the ability of Hsp90 to stabilize survival signaling proteins and hence contribute positively to apoptosis induction. This hypothesis has been supported by the work of Mollerup et al. where a recombinant truncated form of p23 mimicking the cleaved form impaired anti-aggregating activity towards heat inactivated citrate synthase [200]. A recent publication also supports a role for p23 in cell survival. Rao et al. reported that blockage of p23 cleavage reduced ER stress induced cell death [72]. They did not study whether a truncated p23 would enhance such death, but found that immune- or siRNA depletion of p23 enhanced ER stress induced cell death. As ER stress was found to disrupt p23`s interaction with the pro-apoptotic BH3-only protein PUMA, the molecular basis for p23`s protective role could be its binding to PUMA and inhibition of PUMA translocation to the mitochondria where it is known to interact with Bax and trigger cytochrome c release [229]. While the ER stress induced cleavage of p23 was postulated to be the most likely explanation for dissociation of p23 and PUMA, whether the truncated form of p23 was unable to interact with PUMA was not investigated.

In this context it is worth mentioning that Rao et al. only reported the D142-site to be a caspase cleavage site in p23 upon ER stress induced apoptosis. We had to mutate both D142 and D145 in order to totally abrogate cleavage, but our impression was that the primarily cleavage site was at D142. The reasons for this discrepancy could be that while they studied p23 cleavage in transfected cells, we studied the cleavage in *in vitro* cleavage assays with recombinant caspases and with *in vitro* translated p23. It could be that in our system the second site was more exposed and available to caspase cleavage than it would be in a cellular system. Also the presence of two phosphorylation sites for casein kinase II (CKII) close to the cleavage sites could influence the cleavage differentially in the two systems [230,231].

Another possibility for p23 to function anti-apoptotically became evident when p23 was reported to be identical to PGE2 synthase [73]. PGE2 stimulates adenylyl cyclase and the production of cAMP which can modulate apoptosis in hematologic cells [232,233]. We have found that PGE2 and cAMP protect against anthracycline-induced apoptosis in NB4 cells (Wergeland, Gausdal, Kleppe, Døskeland, unpublished results). Therefore the proposed

PGE2 production of p23 could contribute anti-apoptotically. However, results by us (Wergeland, Gausdal, Kleppe, Døskeland, unpublished results) and others [75] question whether the PGE2 synthase activity of p23 is sufficient enough to contribute physiologically. We therefore strongly doubt that the established protective role of p23 in cell survival [72] could be due to PGE2 production.

Discussion Paper II

We report protein modifications resulting in targeting of cap-dependent initiation and stimulation of a non-cap-, probably IRES-dependent translation, stalled elongation and changed mRNA preferences for translation in anthracycline-induced apoptosis. The modifications identified include dephosphorylation events and caspase and/or proteasome-mediated cleavages/downregulation of essential proteins involved in the abovementioned processes. The proteins that we found targeted in this study and their effect on translation and mRNA processing are summarized in Figure 7.

Until recently, relatively little attention has been focused on the changes in protein synthesis that accompany the commitment and execution phases of apoptosis. Induction of apoptosis is in many cases associated with a rapid and substantial, although incomplete inhibition in protein synthesis [102]. As a result of stress-induction, both global and specific protein synthesis are modulated through changes in the phosphorylation status of proteins controlling translation [207], through regulation of the association of these factors into functional complexes and targeted cleavage of factors by proteases [234]. In addition to the hits that we report here, also eIF4G, eIF4B, eIF2-α and the p35 subunit of eIF3 are reported cleaved by capases upon cell death induction [102]. Also the two other ribosomal P-proteins contributing to the stalk formation, rpP0 and rpP1, are dephosphorylated in Fas-induced apoptosis [235]. We did not check specifically if these proteins are modified in our system, but we cannot exclude this. In addition, the polypyrimidine tract binding protein, found cleaved in Fas-induced apoptosis [193] and able to both inhibit [236] and enhance [237] translation of mRNAs with IRES, is totally degraded in anthracycline-induced apoptosis (Gausdal et al., unpublished results obtained in COFRADIC study on IPC-81 cells described in Supplementary Table 3).

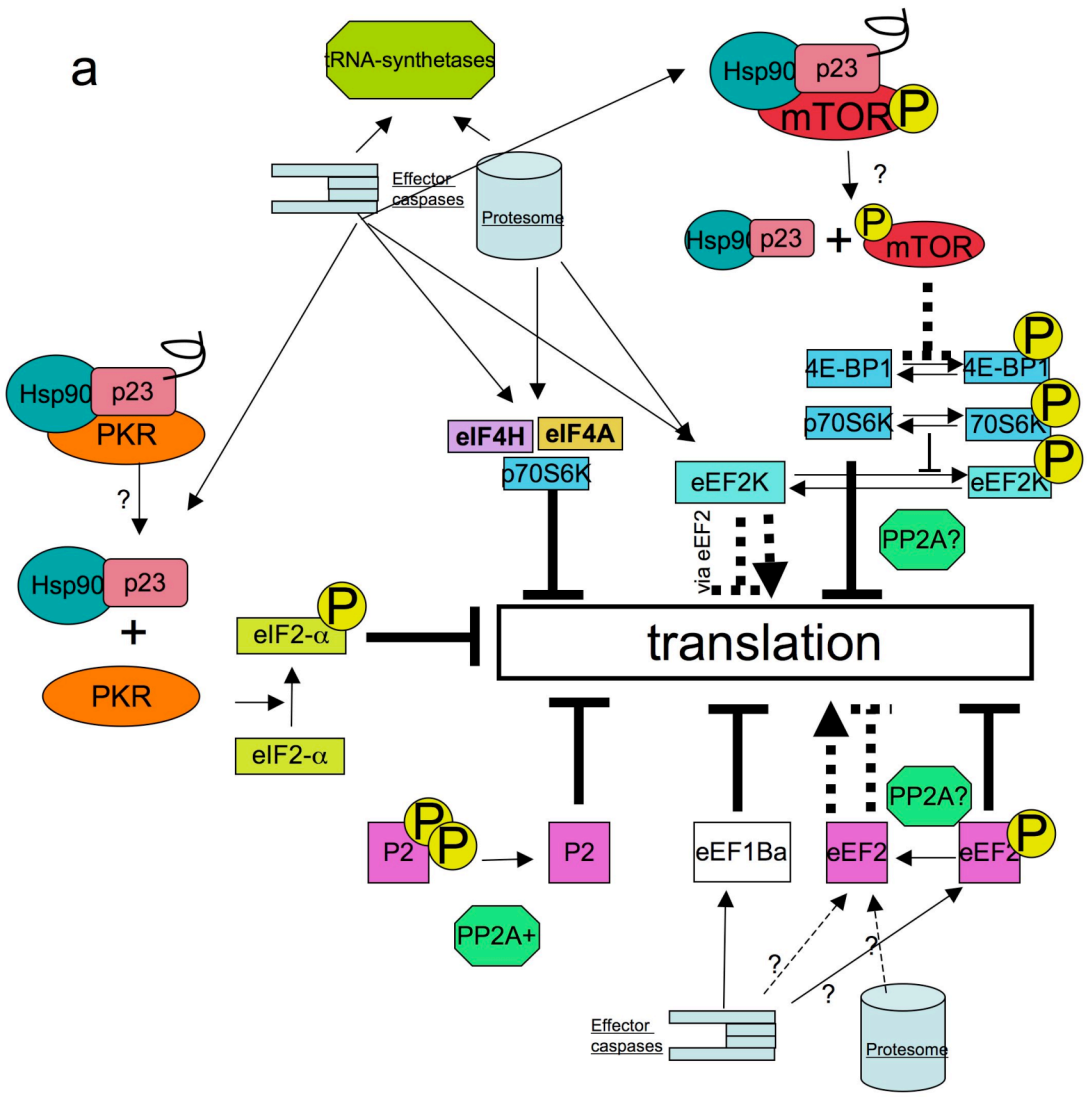
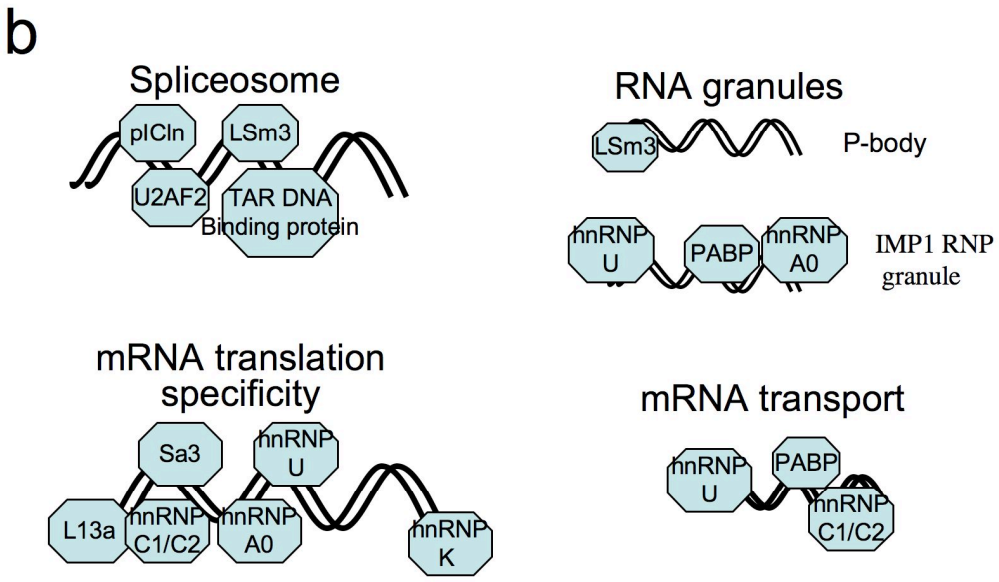


Figure 7. Anthracycline-induced changes in proteins involved in protein synthesis and mRNA processing. (a) Modifications in proteins related to protein synthesis initiation and elongation. Several proteins are targeted by dephosphorylation (rpP2, eEF2, eEF2K, 70S6K, 4E-BP1), caspase-mediated cleavage (p70S6K (Supplementary Figure 2), eEF1Ba, p23), and/or degraded by alternative ways, such as e.g.by the proteasome (eIF4H, eIF4A, eEF2, eEF2K) in anthracycline-induced apoptosis. The dephosphorylation of proteins is probably by PP2A. (b) Next side. Modifications in proteins related to mRNA splicing, degradation/stability, localization and transport. For the specific function of each protein and postulated effect of cleavage/degradation see text, Paper II and Supplementary Table 1.



In addition to the proteins reported in Paper II, the cleavage of p23 might also contribute to the translational switch from a cap- to non-cap-dependent mode. The mTOR signaling pathway is facilitated upon interaction with Hsp90, and GA was found to suppress phosphorylation of the mTOR targets p70S6K and 4E-BP1 and hence inhibit cap-dependent translation [238]. A cleavage of p23 could very likely, as does GA, lead to suppressed phosphorylation of 4E-BP1 and hence inhibited cap-dependent translation. Cleavage of p23 could also facilitate the switch in protein synthesis through increased eIF2-α phosphorylation. Protein kinase R (PKR) is one of the stress-activated kinases that phosphorylates and inhibits eIF2-α [239]. PKR is bound to Hsp90 and its activity is repressed in the Hsp90 complex [85]. Upon p23 cleavage, the ability of Hsp90 to inhibit PKR might be compromised, hence freeing PKR to phosphorylate eIF2-α and stimulate IRES-mediated translation. Interestingly, PKR has been reported to be activated upon caspase cleavage in Fas-induced cell death [240].

To us, one of the most exciting findings was the dephosphorylation and total degradation of eEF2K. The eEF2K was found to have a more than 10-fold downregulation of its N-terminus by COFRADIC analysis. Immunoblotting confirmed the COFRADIC finding and revealed that dephosphorylation of eEF2K marginally preceded its degradation

(Supplementary Figure 3). However, we have not determined if eEF2K is a caspase target or degraded by other means. No stable fragment of eEF2K was detected by immunoblotting, but considering the COFRADIC data and that the antibody used for detection of eEF2K recognizes the N-terminus, we cannot exclude that such a fragment is generated. Using a caspase inhibitor or an antibody towards another epitope would be necessary to determine this. The sequence of eEF2K (725 amino acids) contains several possible caspase cleavage sites with the consensus DXXD [213], and one of these are located to the N-terminus ($D^{30}SDD^{33}$). The activity of such a caspase generated fragment could either be compromised, changed or still intact. The catalytic domain of eEF2K is in its N-terminal half [241], so a cleavage her could interfere with its catalytic activity. Also the phosphorylation sites of eEF2K at serine-residues in the C-terminal would be present in such a fragment. However, cleaved eEF2K would most likely be dephosphorylated as we did not detect a fragment by the use of an antibody toward Ser366 and since p70S6K, that phosphorylates eEF2K at Ser366, was both dephosphorylated and cleaved by caspases. We have not checked the phosphorylation status of the other sites in eEF2K phosphorylated by other kinases [107,242,243], but it is of course possible that these sites will also be influenced by the activity of anthracyclines. Dephosphorylated eEF2K is known to be active, however if a eEF2K lacking 33 amino acids in its N-terminal will be able to phosphorylate and hence inhibit eEF2, we do not know. That eEF2 is incompletely dephosphorylated (Supplementary Figure 3) could support the hypothesis of a still active eEF2K. However, lack of complete dephosphorylation of eEF2 could also be due to inhibited PP2A phosphatase activity towards eEF2 regulated e.g. in an mTOR dependent manner [244,245]. Hence, the effect of drug-treatment on eEF2K is so far not conclusive and needs to be further elaborated.

eEF2 is, when compared to eEF2K, only dephosphorylated to a limited degree upon DNR-treatment and seems to be only marginally degraded or cleaved. To speculate how DNR-treatment affects the activity of eEF2 is therefore not easy, since we do not know if it is the same pool of eEF2 that is both dephosphorylated and degraded/cleaved and since we have so far not studied the nature of the modification. However, we postulate that despite some dephosphorylation, eEF2 is inhibited, at least to a certain degree, through the presence of a dominant phosphorylated eEF2 pool and the limited degradation of

eEF2. This could contribute to stalled elongation and to mediate a cap- to IRES-dependent synthesis switch. In addition to the direct effect on eEF2, proteins affecting eEF2 activity is also modulated (rpP2 dephosphorylation and eEF1Ba cleavage) possibly further resulting in a compromised translocation process and a change in mRNA specificity. Hence, the combined effect of targeting elongation on this level will probably result in a switch in preferred protein synthesis.

We show that DNR, like rapamycin, inhibits cap-dependent protein translation due to inhibition of the mTOR pathway. We saw this by decreased phosphorylation of the mTOR targets 4E-BP1 and p70S6K. Their dephosphorylation was not due to inhibition of upstream activation of mTOR, as mTOR itself is not dephosphorylated. Hence their dephosphorylation is most likely mediated by activation of a phosphatase. The effect of the phosphatase could be enhanced by a compromised activity of P-mTOR, due to e.g. less stabilizing chaperone activity by the Hsp90 complex due to p23 cleavage. In addition to its dephosphorylation, p70S6K is cleaved by caspases (Supplementary Figure 2). The sequence of p70S6K (525 amino acids) contains only one consensus site for caspases, $D^{393}SPD^{396}$. This cleavage, more or less in the middle of the protein, will most likely compromise its activity. However, this should eventually be tested by looking at its activity towards the substrate S6. An interesting notion is that p70S6K also phosphorylates and inhibits Bad [246]. Hence, a dephosphorylaiton of p70S6K would also have a pro-apoptotic effect through activation of Bad.

Between the level of transcriptional and translational regulation, lies regulation on the level of mRNA processing. Results from out COFRADIC study indicate that anthracyclines in addition to exerting translational regulation, also influences mRNA splicing by targeting members of the spliceosome. Other, more indirect events that also affect the rate and specificity of mRNA translation, and hence the proteome, such as accessibility of mRNAs to the ribosomes, transport out of the nucleus and stability of the mRNA in the cytosol are also targeted in anthracycline-induced apoptosis. Several of the COFRADIC findings not discussed in Paper II, point to the fact that these processes are affected and contributes to altered translational specificity in anthracycline-induced apoptosis (Figure 7b and Supplementary Table 1).

Several proteins involved in mRNA splicing are targeted in DNR-induced apoptosis and underscores the spliceosome as a specific target for anthracyclines (Figure 7b). This targeting would affect the production of mature mRNA for translation and could be somewhat specific in its preferences so that mRNAs coding for survival proteins are preferentially matured into translatable mRNA. pICln is part of the methylosome that methylates Sm proteins resulting in assembly of Sm proteins onto U snRNAs to form the core of the spliceosomal snRNPs [247]. pICln might also have a function in snRNP assembly unrelated to the methylation process [248]. A cleavage of pICln could affect assembly of the spliceosome. Also Lsm3 (like-Sm), the RNA binding motif protein 39 isoform a, the U2AF2 splicing factor and TAR DNA binding protein are modified in our study (for references see Supplementary Table 1).

While some mRNAs are programmed for immediate translation, other mRNAs are programmed for delayed translation and stored in granules until environmental or developmental cues call for their translation. Several of the modified proteins we identified are part of different RNA granules. The processing body (P-body) is a cytoplasmic RNP granule that is involved in mRNA decapping and decay [249]. Lsm proteins, known to function in pre-mRNA splicing are present in P-bodies and also function in mRNA decay [250]. The IMP1 RNP granule, is a newly identified granule thought to function as a post-transcriptional operon containing among others proteins several hnRNPs (i.e. A0 and U) and PABP2 [124]. These proteins involved in mRNA modification and accessibility are modified in DNR-induced apoptosis and could be a mode for regulating the mRNA pool available for translation. Another process governing mRNA availability for translation is transport out of the nucleus, and proteins such as PABP and members of hnRNPs involved in mRNA transport are also cleaved or degraded. Also proteins that regulate translation of specific mRNAs are modified in this study including several hnRNPs and S3a and L13a (see Supplementary Table 1 for references and details).

The hnRNPs are a diverse group of pre-mRNA/mRNA binding proteins found both in the nucleus and in the cytoplasm that associates with mRNA transcripts and influence their function and fate. In addition to their role in pre-mRNA processing and splicing

[251], they also play important roles in the control of specific mRNA translation, transport and stability. Several hnRNPs are modified in response to DNR-induced apoptosis, and for their specific function, I refer to Supplementary Table 1.

Induction of survival protein synthesis and synthesis of ribosomal proteins

In this study we show that cells treated with anthracyclines have preferred synthesis for proteins that are involved in protein synthesis itself and in promoting survival to the cell. We postulate that the preferred synthesis is manifested in the pre-apoptotic phase of the cell death induction. We base this on the facts that several of the changes we found does not primarily serve to totally shutdown all protein synthesis, but rather to switch the preference of proteins synthesized from a general one to a specific one, and the fact that a protein synthesis inhibitor administered one hour after DNR were able to enhance the effect of DNR. The molecular targets we have identified (Figure 7) indicate a switch in protein synthesis from a cap-dependent to a non-cap-dependent. This is probably an IRES-dependent synthesis. However, we cannot say this for sure as we have not proven this directly, and since it is not possible to identify an IRES-sequence in the 5'-UTR of a protein simply by looking at the nucleotide sequence since the IRES is a structural motif.

Several genes involved in cell growth, proliferation, differentiation and the regulation of apoptosis contain IRES elements in their 5'-UTRs and can be translated in an IRES-mediated way. This mode of translation provides a means of escaping a global decline in protein synthesis, and allows the selective translation of specific mRNAs. This indicates that the selective regulation of IRES-mediated translation is crucial to the regulation of cell death and survival. Selective translation of proteins may contribute to the survival of cancer cells under stressful situations, such as lack of nutrients, hypoxia, or therapy-induced DNA damage [88,117]. Also apoptotic control in transformed cells occurs via qualitative rather than quantitative changes in protein synthesis is mediated by a dynamic interplay between cap-dependent and cap-independent processes [252].

We found an upregulated synthesis of rpP2, PDI/P4HB, PCNA and calreticulin. Since we only focused on a small part of the proteome, several other survival proteins in addition to the ones we found are also most likely preferentially synthesized in pre-apoptotic cells

and will contribute to the drug-induced anti-apoptotic response. How might the proteins we identified promote anti-apoptotic functions? The presence of de-phospho rpP2 (both due to dephosphorylation and increased synthesis of un-phospho rpP2 combined with a slow phosphorylation rate) will influence the fidelity and selectivity for mRNA transcripts [253] and could facilitate translation of mRNAs coding for proteins important in anti-apoptotic functions and not highly translated under normal conditions. Proliferating cell nuclear antigen, PCNA, is a cofactor for DNA polymerase delta, a polymerase with 3'- to 5'- exonuclease activity and proof reading activity [254]. In response to DNA damage, PCNA is ubiquitinated and is involved in the RAD6-dependent DNA repair pathway [255]. PCNA has a proliferating effect and has been found to be upregulated in several cancers [256]. siRNA knock down of PCNA led to downregulation of apoptotic inhibitors and upregulation of pro-apoptotic genes. This makes it understandable that a stress-induced upregulation of PCNA could have an anti-apoptotic effect in anthracycline-treated cells. PDI functions among other things as a chaperone and inhibits aggregation of misfolded proteins [257], and PDI upregulation has been demonstrated to protect against neuronal cell death [258]. Calreticulin is an ER chaperone and is involved in "quality control" within secretory pathways [259]. In addition, calreticulin has also been shown to influence translation of several mRNAs. It blocks translation of p21 mRNA and hence promotes proliferation [260]. It also represses translation of growth-inhibitory C/EBP proteins [261]. However, the effect of translational upregulation of calreticulin is not definite, as calreticulin is translationally downregulated in Ras-transformed cells [262], induced in fenretinide-induced apoptosis [263] and triggers an immunogenic cancer cell death [209].

Reports have stated that following stress, ER serves as the primary compartment for the synthesis of soluble and secretory proteins and that ER-bound ribosomes provide a unique mRNA translational function [264-266]. We show that both rpP2 and calreticulin are preferentially synthesized in DNR-treated cells, and others have reported their translocation to the cell surface upon death induction [209,235]. Whether the anthracycline-induced protein synthesis we report here is preferentially located to the ER, we cannot conclusively state from our TEM experiments. However, several ribosomes seemed to

still attach to the ER even though polyribosomes were disaggregated in the cytosol, so this is plausible.

Anthracycline-induced cell death and protein synthesis inhibitors

Addition of a protein synthesis inhibitor is often essential for the ability to induce cell death via death receptors. This has been ascribed to the activation of survival pathways through NF-kB mediated gene-transcription and synthesis of proteins with an anti-apoptotic function [55]. Also DNR induces translocation of NF-kB to the nucleus [267]. When present for a prolonged period (>15 h) of time, DNR has been reported to affect the expression of up to 10% of the genes expressed in some cells [268,269]. Many of the genes upregulated by DNR codes for survival-associated proteins, presumably because DNR activates survival signaling through e.g. the NF-kB and PI3-kinase pathways [30]. We can not completely exclude that a part of the survival protein upregulation due to DNR-treatment to some extent is due to transcriptional regulation and not only to translational regulation. However, we have shown that DNR does not significantly influence gene transcription, and that all but one protein (PCNA) of the *in vitro* translated proteins in the sub-proteome analyzed (pI 4-5), showed a more than two-fold change in expression after DNR treatment (Supplementary Table 2). To study proteins specifically upregulated transcriptionally by DNR, a proteome analysis of cells after prolonged exposure to moderate concentrations of DNR would probably be a suitable approach and could reveal the identity of may proteins generally induced in stressed cells [270]. However, in our experimental setup, we concluded, based on mRNA levels and protein modifications in the protein synthesis machinery, that the induction of survival protein synthesis that we see is primary due to translational regulation of protein synthesis.

We report in this paper that inhibition of protein synthesis in the pre-apoptotic phase of anthracycline-induced apoptosis significantly enhances cell death both in cell culture and in animal models of AML. We postulate that the enhancing effect of the protein synthesis inhibitor on anthracycline-induced cell death is due to shutdown of the pre-apoptotic drug-induced synthesis of survival proteins such as e.g. chaperones.

Previous reports on the effect of CHX on cell death are somewhat contradictory, and CHX has been reported both to protect against [271,272] and to enhance [273,274] apoptotic cell death (see also Introduction). The reasons for the discrepancy between these reports are several, and the effect of CHX appears to depend on the cellular system, the concentrations used and the administration schedules. That CHX prevented cell death lead to the assumption that *de novo* protein synthesis of "killer" proteins was required for apoptosis to occur [275]. However, the concentrations of CHX used are crucial for its effect and low doses of CHX that only modestly or transiently decrease protein synthesis has been found to induce cytoprotective signaling pathways and upregulate anti-apoptotic proteins such as Bcl-2 [276]. Therefore, the use of low doses of CHX and a consecutive induction of survival proteins can explain some of these reports [277]. Also the fact that CHX induces apoptosis alone [58,274] argues against the requirement for *de novo* synthesis of "killer" proteins, and rather indicates that the machinery necessary to induce apoptosis is constitutively expressed in a latent form and that synthesis of labile short lived repressors/survival proteins are required to prevent the dormant apoptotic apparatus from inducing apoptosis [278,279]. In addition, the fact that CHX is a reversible inhibitor of protein synthesis, primes for caution when interpreting the results of experiments that are preformed over an extended period of time as sustained inhibition of protein synthesis might not be achieved [277]. Our studies were carried out with doses of CHX that more or less completely inhibited protein synthesis (measured by [^{35}S]-methionine incorporation) during the experimental readout. After prolonged exposure, CHX will itself induce apoptosis [58]. However, CHX alone did not induce significant apoptosis within the time-course of our experiments. We therefore conclude that its inherent apoptosis-inducing ability is not relevant for the results obtained in this study.

The time at which the protein synthesis inhibitor is supplemented relative to the other drug is also an aspect. We found that the death enhancing effect of CHX was most prominent when it was administered 1-1.5 h after the anthracycline. In a majority of the reports where CHX is reported to inhibit cell death, CHX is administered prior to the drug. CHX will shut down the synthesis of cyclines that are essential to pull a cell through the cell cycle. Hence, CHX induces cell cycle arrest. Drugs that are only effective e.g. in transtition between phases or in a particular cell cycle phase will

therefore most likely be inhibited by the presence of CHX given before addition of the drug. This is illustrated in the case of camptothecin-induced cell death, a DNA topoisomerase I inhibitor that acts only in the S-phase [58]. Although anthracyclines exert their toxic effect in all phases of the cell cycle, the fact that they are topoisomerase II inhibitors indicate that cells in the S-phase are more sensitive to drug-induced apoptosis [280]. Stalling of cycling cells by protein synthesis inhibitors and thereby denying the drug access to cells in S-phase, could explain an antagonistic effect of CHX found by many.

Finally, the finding that elevated drug-doses induce a "frozen" apoptotic morphology can also explain some of the reports where elevated drug and/or CHX concentrations have been used, since high drug doses prevent cells from displaying classical morphologic features of apoptotic cell death.

We did not only observe an effect of CHX in cell culture, but saw the same effect in animal models of AML. One possible explanation for the death-enhancing effect of CHX observed in animal studies could simply be increased toxicity of the anthracycline owed to increased and stressed drug metabolism in the liver due to the presence of CHX. However, this unspecific effect of CHX is unlikely as others reported, when looking at apoptosis in the murine intestinal tract of *in vivo* treated animals, that CHX administered together with adriamycin/doxorubicin inhibited the effect of the anthracycline [37]. This argues against that CHX increases the toxicity of the anthracycline via stressed drug-metabolism in the liver. CHX could also have influenced uptake of the anthracycline and thereby increased the toxic effect of the drugs by enhancing its intracellular concentration. However, CHX has been reported not to influence the uptake of doxorubicin in leukemic cells [36].

While the abovementioned can easily explain most of the discrepancy found in the literature on the effect of CHX on cell death, the data reported by Furusawa et al. that quite contrary to our data states that CHX abrogates the effect of doxorubicin in a leukemic mouse model, is not easy to explain [36]. The discrepancy between these two works must be due to experimental differences. In the two studies, different cell lines were used to induce leukemia and different rodents were used as receivers. While we inoculated the leukemia cells via the tail vein, they inoculated the cells i.p. This could

result in leukemias with somewhat different qualities. While they administered CHX prior to the anthracycline, we administered it 1-1.5 h after. However, they administered a high dose of CHX (15 mg/ml for mice compared to our 5 mg/ml) that should be sufficiently high not to induce survival protein synthesis [276]. While we administered the anthracyclines i.v. or perorally, they gave the drugs i.p. This could differentially influence both the effective concentrations of the drug and the drug metabolism in the liver.

Ribosomal Protein P2

Both CK II [109] and G protein-coupled receptor kinase 2 (GRK2) [281] phosphorylate rpP2. Which kinase that are the predominant one, is not known. Hasler and coworkers found that *in vivo,* the CK II only phosphorylated rpP2 at Ser105, and postulated this to be the primarily site of phosphorylation. However, we have shown that newly synthesized rpP2 is phosphorylated in a consecutive manner from a non- to a mono- and di-phosphorylated form. Consequently, the Ser102 site is phosphorylated to a similar extent since the main form of rpP2 in non-apoptotic cells is a singel spot with pI correlating to a di-phospho form of rpP2. We have not investigated whether there is a preference in order of phosphorylation between the two sites or whether specific kinases show any preference toward the two phosphorylation sites. The kinetics of the phosphorylation is rather slow and not complete within 1 h. This is in contrast to what has been reported in yeast, where rpP2 phosphorylation reaches saturation within 10 min [282]. This discrepancy could be due to cell specific variations. The slow phosphorylation of rpP2 could be a way of regulating peptide elongation by governing the availability of di-phospho rpP2 to interact with eEF2.

We found di-phospho rpP2 to be dephosphorylated by a calyculin A sensitive phosphatase. Also the MCF-7 mammary carcinoma cell line show dephosphorylation of rpP2 upon anthracycline treatment indicating that this is not an AML specific event (Supplementary Figure 4a). rpP2 seems to de dephosphorylated in a consecutive manner, however, we have not looked into whether there is a preference to the sequence of dephosphorylation or whether it is completely random. rpP2 has previously been reported to be dephosphorylated in Fas-induced cell death in a caspase dependent manner [235].

Contrary to our finding, only one dephosphorylation step was noted. Their finding indicates that the phosphatase activity could be regulated at least partially by caspase cleavage. The phosphatase most likely responsible for rpP2 dephosphorylation is PP2A [283]. PP2A is activated early in Fas-induced apoptosis in neutrophiles [284] and caspase cleavage of PP2A results in increased PP2A activity towards specific pro-apoptotic substrates involved in the cellular apoptotic response [285].

The dephosphorylation of rpP2 also happened in AML patient blasts after exposure to anthracyclines. The % dephosphorylation differed within the FAB sub-classification groups of AML. When the level of anti-apoptotic Bcl-2 was determined in patient blasts, there was a correlation between rpP2 dephosphorylation and Bcl-2 level, where high levels of Bcl-2 inhibited rpP2 dephosphorylation (Supplementary Figure 4b,c). Interestingly, also in IPC-81 cells stably transfected with Bcl-2 and completely resistant to anthracycline-induced apoptosis, rpP2 dephosphorylation was inhibited (Supplementary Figure 4d). Whether the inhibition is by direct interaction between Bcl-2 and PP2A, or whether the inhibition of PP2A activity is secondary and a result of general Bcl-2-mediated inhibition of apoptosis and caspase activation, we cannot say.

De-phospho rpP2 interacts badly with eEF2 [111], and the GTPase activity of eEF2 and translocation of peptidyl-tRNA from the A- to the P-site of the ribosome is dependent upon the presence of rpP1 and rpP2 on the large ribosomal subunit [108,111]. However, inactivation studies in yeast have shown that the P1 and P2 proteins are not absolutely essential for ribosome activity, but are able to affect the specificity of translation of some specific mRNAs [253]. It has been shown that the cytoplasmic pool of rpP2 are interchangeable with the phosphorylated rpP2 pool present on the ribosomes [253]. The dephosphorylation and enhanced synthesis of rpP2 combined with a slow phosphorylation rate may contribute to shifting the pool of rpP2 present on the ribosomes to a de-phosphorylated one and thus facilitate both a slowing of elongation, that might result in IRES-dependent translation [105], and a change in mRNA specificity for translation.

Several reports point to the utilization of rpP2 as a target in cancer therapy. Our finding that rpP2 is dephosphorylated early in apoptosis supports this notion. Monoclonal antibodies against ribosomal P-proteins have been shown to penetrate into several cell lines and induce apoptosis [286]. Antibodies directed against the conserved C-terminal sequence of the P-proteins inhibited protein synthesis elongation by blocking access of elongation factors to the ribosomal stalk [287]. Others have shown that antibodies towards the P-proteins have an inhibitory effect on global protein synthesis [288]. The gonadotropin-releasing hormone (GnRH) analogues are used in the treatment of several cancers, and inhibit proliferation by decreasing the rate of protein synthesis through downregulation of rpP1 and rpP2 [289]. The drug trichosanthin interacts specifically with rpP2 [290], and has been shown to have anti-tumor effect [291].

Anthracyclines and autophagy

An interesting notice in view of the mimicry by DNR of rapamycin actions was the lack of appreciable autophagy induction by DNR. Electron microscopy did not reveal any obvious signs of autophagy. Also the fact that disaggregated ribosomes remained numerous late in apoptosis indicates no induction of autophagy as ribosomes are among the first targets in autophagic cell death [292]. Autophagy can provide nutrients and energy to stressed cells [211], and is stimulated by mTOR inhibitors such as rapamycin, in part through the activation of eEF2K [212]. The lack of obvious autophagy may be due in part to the near complete downregulation of eEF2K in DNR-treated cells.

Drug-induced surface expression of proteins: A role for NAC?

The finding that nascent polypeptide-associated complex (NAC) is cleaved at $EEQD^{42}$ by caspases is tempting to link to the increased surface expression of proteins such as calreticulin an rpP2 that occur upon apoptosis induction [209,235]. NAC was also reported cleaved in Fas-induced apoptosis, but the cleavage site was not identified [223]. NAC is among the first ribosome-associated proteins to bind nascent polypeptides as they emerge from the ribosomes. The exact role of NAC is debated (for review see [293]), however it has been postulated that NAC functions as a negative regulator of translocation of proteins

into ER [294,295]. A cleavage of NAC could compromise this ability and allow ER-targeting of proteins not originally destined for ER and/or secretion.

Discussion Paper III

In this paper, we report anthracycline targeting of the endocytic machinery resulting in a decreased transferrin endocytosis and an accumulation of surface receptors. This was seen both in cell lines and in blasts from patients receiving induction treatment.

CLC, AP2-α and Eps15

Anthracyclines induced cleavage and degradation of several proteins involved in clathrin-mediated endocytosis. Two of the proteins we found to be targeted upon cell death, CLC and AP2-α, were not exclusive caspase targets as the presence of zVAD did only partially inhibit their degradation. CLC contains the putative caspase target sequence $D^{73}AVD^{76}$, so its cleavage by caspases is feasible. CLC disappeared before visible caspase activation, but caspases have been shown to be active pre-apoptotically [296]. Whether mutating the caspase target sequence would abrogate degradation, was not tested. However, mutating the destruction box (D-box) motif [297] in the C-terminal tail of CLC, abrogated CLC degradation. Which mode of CLC degradation that is the prominent one, we do not know, but our mutation analysis points to the proteasome as an important player. This shows that even though anthracyclines bind to and inhibit the proteasome [298] and that several proteasome subunits are targeted for cleavage in IDA-induced apoptosis (Gausdal, Døskeland, et al., unpublished results), the proteasome is active at least in the induction phase of anthracycline-induced apoptosis and able to mediated degradation of proteins. We have not yet investigated which ligase that are involved in CLC ubiquitinylation, but one ligase, cbl, that is involved in ubiquitinylation og EGFR could be a candidate as it is found in the vicinity of clathrin coated pits [299].

The modification in AP2-α was also only partially inhibited by the presence of zVAD, but totally inhibited by a proteasome inhibitor. We therefore concluded that AP2-α is not a sole caspase target. However, the presence of a stable product of AP2-α at about 50

kDa was puzzling since degradation by the proteasome does not normally result in a stable product, but in complete degradation of the protein. Interestingly, Austin et al. reported AP2-α to be cleaved by initiator caspases in death receptor-induced apoptosis [221]. The cleavage fragment obtained for AP2-α in TRAIL-induced apoptosis is similar to the one we find in DNR-induced cell death, indicating that AP2-α is most likely cleaved at the same site by the two death-induction modes. This could indicate that the proteasome has caspase-like qualities and is able to cleave/partially degrade proteins and leave stable cleavage fragments.

Eps15, originally identified as a substrate for the kinase activity of EGFR (reviewed in [300]) and involved in ligand regulated, but not constitutive endocytosis [136], was also a caspase target. The antibody used for Eps15 detection binds to an epitope in the C-terminal part of Eps15 that is involved in AP2-α binding. The sequence of Eps15 contains several putative caspase target sequences, one of them is in the C-terminal ($D^{795}SPD^{798}$) in the middle of the immunogen (amino acid 694-888). By using this antibody, we were not able to show a stable cleavage fragment of Eps15 indicating that this part of Eps15 is cut off. A cleavage of Eps15 in this area would probably interfere with its binding to AP2-α and generation of the clathrin lattice.

An aspect that we have not addressed in this study and that could play a role both in the function of endocytic proteins and their susceptibility to cleavage/degradation, is phosphorylational regulation. Both phosphorylation of CLC b-chain [301] and AP-2 [302] have been reported to regulate CCV formation [303]. Whether dephosphorylation of these proteins follows apoptosis induction is not known, but AP2-β is dephosphorylated in a PP2A dependent manner [304] and PP2A is a phosphatase activated in apoptosis [285]. We have shown that a calyculin A sensitive phosphatase, probably PP2A, dephosphorylates rpP2 pre-apoptotically upon anthracycline treatment (Paper II). It is therefore not unlikely that proteins involved in clathrin-mediated endocytosis is also affected by this phosphatase and that the pre-apoptotic shutdown of endocytosis that we see could also partly be due to changes in the phosphorylational levels of proteins. CLC is phosphorylated by several kinases, among them CKII [301,305,306]. CKII phosphorylates several proteins that are targeted for caspase-mediated cleavage during apoptosis, and

dephosphorylation of these CKII sites enhances caspase cleavage [230]. Even though we found the degradation of CLC to be mainly via the proteasome, caspase inhibitors abrogated degradation to a certain extent, pointing to an involvement of caspases or to a caspase-like action of the proteasome. A pre-apoptotic dephosphorylation of CLC could also, in addition to affect its activity in CCV formation, increase its susceptibility to degradation. This regulation of caspase mediated cleavage by CKII is also probably seen with p23 (Paper I), as p23 is also phosphorylated by CKII [231].

The notion that CLC is exposed to the cytoplasmic face of the vesicles and interacts with regulatory elements [307,308], is regulated by phosphorylation and binds to Ca^{2+} [309] and calmodulin [310] implicates that CLC also plays a role in signaling. Early targeting of CLC might therefore in also serve a regulatory function in the pre-apoptotic period by selectively facilitating endocytosis of specific receptors and modulate receptor signaling.

Anthracyclines and iron

In this work we used constitutive internalization of transferrin (Tf) as a measure for clathrin-dependent endocytosis. Tf binds Fe^{3+} (Fe in its toxic form) and is internalized via interactions with the TfR. Acidic conditions in the endosomes facilitate release of iron from Tf, and Tf is recycled together with TfR to the cell surface where it its released [152]. The iron-interacting quality of the anthracyclines could be an issue in this context, as interactions with iron is involved in drug-induced ROS formation [24], and reduction of iron uptake has been shown to decrease the apoptotic inducing effects of doxorubicin [311]. Especially in the heart, iron chelators have been shown to be cardioprotective of the cytotoxcic effect of the anthracyclines [312]. Due to the iron interacting mode of the anthracyclines, this classic model for measuring endocytosis is maybe not optimal when used in combination with anthracyclines. Nevertheless, at the anthracycline concentrations that were used in this study, involvement of ROS generation for oxidative DNA damage is probably not a major issue [24].

The sensitivity of tumor cells to undergo apoptosis upon iron deprivation differs, but cells of hematopoietic origin are sensitive to iron deprivation and undergoes apoptosis [313,314].

However, cells contain an intracellular storage or iron sufficient to sustain immediate metabolic needs [315]. This, in addition to the fact that we in our experimental setup use medium with abundant iron supply and have short death-induction times, argues against changes in intracellular iron concentration as playing an important role in DNR apoptosis-induction. However, for patients receiving chemotherapy with prolonged exposure to the drugs and with possibly limiting iron supply, the decreased uptake of iron could have an enhancing effect on anthracycline-induced apoptosis. It is therefore interesting to see that transferrin uptake was decreased in AML blasts isolated from patients that receive induction therapy.

Surface receptor accumulation and signaling

We have not specifically investigated whether the drug-induced decrease in transferrin uptake and the accumulation of surface receptors represent an absolute shutdown of all modes of endocytosis or whether some clathrin or clathrin-independent internalization is still going on. Late in apoptosis induction and in the execution phase, probably all endocytic activity are more or less completely compromised due to complete degradation and cleavage of several proteins. However, effects on endocytosis in the initial phases of apoptosis are probably more subtle and it its these changes that are the most interesting to study. We have mainly focused on the clathrin-mediated endocytosis of receptors since the proteins we found targeted are primarily involved in this process. We also speculate that clathrin-independent internalization is targeted due to degradation of e.g dynamin and Eps15, but we have not tested this. However, internalization modes independent upon dynamin exists [316], receptors can be internalized by different modes of endocytosis [317] and endosomal sorting have different requirement for clathrin [318]. We have not investigated the effect of drug-treatment on each specific mode of internalization to compare whether e.g. IFNγ-R are internalized via caveloae when clathrin-mediated endocytosis is compromised. Due to this, we cannot exclusively conclude from our data that only surface signaling from IFNγ-R mediates the death-enhancing effect, as some IFNγ-R could be internalized due to other means of endocytosis.

Cell culture studies showed that upon IDA treatment, Fas-R and IFNγ-R accumulated on the surface in cells with reduced tranferrin endocytosis. These receptors are mainly considered death-receptors even though the effect of IFNγ treatment can differ depending on the cell system and the presence of other cytokines [319]. Activation of Fas-R and IFNγ-R enhanced apoptosis in HL60 cells treated with DNR. We postulated therefore that the accumulation of these receptors together with a compromised STAT5 signaling could contribute to apoptosis induction. However, the picture is not quite this simple. Also the stem cell factor (SCF) receptor c-kit accumulated on the surface upon drug treatment (Supplementary Figure 5a). Whether co-administration of SCF inhibits anthracycline-induced apoptosis has so far not been tested, but it is plausible as c-kit is an oncogene [320], SCF is considered a survival promoting cytokine and treatment with anti-SCF enhances anthracycline treatment [321]. GM-CSF-R, another growth-promoting receptor, did not accumulate on the surface of cells (Supplementary Figure 5b), and treatment with GM-CSF did not modulate apoptosis (Supplementary Figure 6). However, STAT5 signaling from GM-CSF-R was also compromised in IDA-treated cells that showed a reduced transferrin uptake (Supplementary Figure 7). The reason why we do not see an accumulation on GM-CSF-R could be due to low endogenous expression of the receptor. We also saw an accumulation of the RTK Flt3 on AML blasts isolated from patients receiving induction treatment (Supplementary Figure 8). However, we were not able to reproduce this effect on AML cell lines something that could reflect a higher expression of Flt3 on primary blasts. The effect of surface accumulation of a specter of receptors on blasts in patients receiving chemotherapy is not clear and will be influenced by the presence of ligands and signaling molecules in the complex extracellular matrix and on the surface of neighbouring cells. Especially, to predict the effect of IFNγ-R accumulation is not straightforward and will probably vary since the effect of IFNγ on human AML blasts is diverse when it comes to proliferation inhibition or apoptosis induction and depends on the local cytokine network [319].

The reduction of anti-apoptotic STAT5 signaling could contribute to death induction. However, also pro-apoptotic STAT1 activation [322] from IFNγ-R was abrogated upon IDA treatment (Supplementary Figure 9). Therefore both death- and survival promoting signaling from the receptor is affected. The effect of interfering with STAT signaling is

not clear. STATs are constitutively activated in AML [323], and depending on the stability of the mRNA and proteins regulated by the STATs, the effect of turning off STAT signaling might not be manifested immediately. Abrogation of survival signaling itself is probably not enough to modulate DNR-induced apoptosis, as GM-CSF did not have an enhancing effect on DNR-induced apoptosis even though we saw compromised STAT5 activation also from GM-CSF-R after drug treatment. This indicates that pro-apoptotic signaling, probably independent upon internalization, is still going on from the IFNγ-R, and that IFNγ-R also exerts its effects via other mechanisms than just through activation of STATs.

Receptor-mediated drug delivery

The use of targeted delivery of liposomal anthracyclines depending on transferrin or folate receptor endocytosis for internalization have been reported to successfully induce apoptosis in cells [23,154]. This could be due both to uptake of the drug by internalization before targeting and shutdown of the endocytic machinery, uptake of the drug by other means than receptor-mediated endocytosis or due to release of the drug from membrane bound liposomes followed by uptake of the released drug into the cell [23]. However, even though initial delivery of liposomal anthracycline by endocytic internalization has proven efficient, uptake of the drug by this pathway after initial induction of apoptosis will probably be compromised. Based on this, we therefore question the use of combining an anthracycline with a second drug that is dependent upon receptor-mediated internalization for its activity.

Anthracyclines and intracellular trafficking

Apoptosis has by others previously been reported to be accompanied by changes in the early secretory pathway between ER and Golgi [324,325] and loss of cisternal organization of the Golgi due to caspase-mediated cleavage or essential proteins such as syntaxin 5, giantin and Golgi stacking proteins [326]. By an immunoblot screen, we had identified EEA1, involved in early endosome fusion, as a caspase target. Early endosome fusion has previously been reported to be blocked in cytochrome c induced death by caspase-

mediated cleavage of rabaptin-5, an effector of Rab5 [327]. The COFRADIC study reported in Paper II showed that VAMP-3, involved in recycling endosome fusion and secretion [328] was downregulated in anthracycline-induced cell death.

In a second COFRADIC on IPC-81 cells treated with IDA and bortezomib, we found a number of proteins involved in intracellular trafficking and proteins linking the endocytic machinery to the cytoskeleton to be targeted for cleavage/degradation (Supplementary Table 3). Vacuolar protein sorting-associated protein 33A (r-vps33a) [329] and VAMP-8 [330] involved in late endosome fusion was found to be cleaved. Fusion of recycling endosomes and secretion were targeted due to VAMP–8 [328] and snapin [331] cleavage. Secretion and exocytosis were also targeted through the cleavage of Munc13-1, which plays an essential role in vesicle priming before exocytosis [332].

Intersectin-1 was also cleaved upon drug treatment, and its cleavage can affect several trafficking steps. Intersectin-1 is a multifunctional protein involved both in clathrin mediated endocytosis as a scaffold protein binding to Eps15 and dynamin [333], in exocytosis through its interaction with SNAP-25 [334], in actin dynamics [335], in signal transduction pathways [336-338] and as a negative regulator of apoptosis [339]. On a more general level, trafficking is targeted by cleavage of SNAP-29, a protein able to bind to a broad range of syntaxins [340], and by the degradation of dynamin and CLC (Paper II) as clathrin coated vesicles are also involved in intracellular trafficking [341].

The Rabs and SNARES control the steps of initial docking/tethering and specificity/fusion between vesicles and intracellular compartments. While several SNARE-proteins came up as hits in the COFRADIC study, we saw no data on the Rabs. An immunoblot screen of several Rabs revealed that only Rab1 was affected, and then only to a limited extent, at the stage in apoptosis when several proteins involved in endocytosis were cleaved (Supplementary Figure 10). However, at prolonged incubation with DNR, also Rab4 and 6 were affected. This shows that the Rabs are stable throughout the induction-course of apoptosis and that the Rabs are not major targets in anthracycline-induced apoptosis

The involvement of the cytoskeleton in receptor-mediated endocytosis has been established [342,343]. Several proteins related to the cytoskeleton were modified upon

apoptotic induction. Spectrin and ezrin are cleaved by proteases. Both link receptors to the sub-membrane actin fibers [344] and internalization of Fas-R is dependent upon redistribution of the receptor to microdomains in the membrane where the actin cytoskeleton is linked to the membrane in an erzin-mediated association [345]. Paxillin is also targeted and links membrane receptors to the underlying cytoskeleton and is in addition involved in signal transduction [346]. Also the degradation of CLC b-chain might interfere with assembly of the cytoskeleton as the CLC b-chain shows regulatory activity towards a microtubule associated protein phosphatase and inhibits its activity [347].

It is clear from our data that anthracycline-induced apoptosis leads to the cleavage/degradation of proteins involved in trafficking and secretion. We have not investigated how seriously the simultaneous targeting of several proteins involved at different stages in trafficking affects intercellular membrane trafficking or how early in the apoptotic scheme these modifications are evident. However, upon treatment with anthracyclines, pre-apoptotic translocation of calreticulin from ER to the cell surface has been reported [209]. We also postulate that the accumulation of surface receptors (Paper III) is a result of endocytotic shutdown combined with induced or sustained recycling of receptors to the surface. This shows that transport from ER to the cell surface membrane is operative in the early phases of apoptosis indicating that the effect on intracellular trafficking either is manifested in the execution phase of apoptosis, that the disruption of proteins involved is not serious enough to completely abrogate exocytosis in the pre-apoptotic phase (we have so far not tested the extent of cleavage of any of these proteins by immunoblotting), or that the protein modifications observed serve to regulate or finetune the exocytic process to facilitate the export of some proteins rather than shut down trafficking per se. A thing to notice is that CLC is also involved in vesicle transport between intracellular compartments [348]. Expecting that the entire intracellular pool of CLC are degraded at the same kinetics, pre-apoptotic degradation of CLC would also affect the formation of CCVs from the endosome and Golgi. However, this is at least not enough to totally impair the secretory pathway even though it might slow it down. Another interesting finding in this matter, was that tomosyn-1, an inhibitor of exocytosis [349] was also cleaved. The cleavage of tomosyn-1 might in some way counteract the effects of protease cleavages of other proteins.

Concluding remarks

When we started this project, we mainly had the following goals: To learn more about the basic mechanism of action of the anthracyclines, to identify possibly new protein targets for directed therapy and detect "Akilles heels" in the ways the anthracyclines work to suggest combination therapy. Our open-ended approach showed that the anthracyclines target numerous pathways/proteins in the cell, including pathways/proteins that have already been exploited and tested for specific drug targeting in the treatment cancers (e.g. Hsp90, the proteasome, cap-dependent protein synthesis). This convinces us that our approach is a successful one when it comes to searching for new drug targets.

We identified several proteins that were targeted for proteolysis/degradation or phosphorylational modifications that had not previously been reported to be modified during apoptosis. Several of the anthracycline-induced changes we found had previously been reported to be implicated in apoptosis induced by other drugs, such as e.g. the cleavage of p23 and several of the proteins related to protein synthesis and endocytosis, but not in anthracycline-induced apoptosis per se. In addition to identifying new single molecular targets, we also found evidence that whole cellular systems are specifically targeted in apoptosis. This accounts for protein translation, clathrin-mediated endocytosis and intracellular trafficking.

We also found that anthracyclines induce a non-cap-dependent stress-induced synthesis of survival proteins following anthracycline treatment and that this could be inhibited by careful and timed addition of protein synthesis inhibitors. This combination of drugs increased AML cell death and increased survival in animal models with AML. Since a general protein synthesis inhibitor like CHX is probably not suitable for use in cancer therapy due to serious side effects, finding ways to selectively perturb the IRES-dependent protein synthesis in anthracycline-stressed AML cells and thereby achieve the same effect as with CHX, but with less side effects, is the aim for further studies. Several of the proteins that we identified as targets in anthracycline-induced apoptosis could be possible targets for intelligent drugs, e.g. rpP2 and eEF2K.

Future perspectives

The open-ended approach that we took to explore anthracycline action opened up several new fields of research to us that requires further investigation.

1. The role of truncating p23 on apoptosis induction will have to be explored. p23-constructs mimicking the truncated form and an uncleavable version of p23 have been generated for transfection studies. Studies are initiated to look at the effect of p23 truncation on apoptosis-induction and on Hsp90 client protein stability.
2. Reports that proteins are translocated to the surface during anthracycline-induced apoptosis and our finding that several proteins involved in intracellular trafficking are modified in anthracycline-induced apoptosis claims for a study of what happens to the secretory/exocytic pathway during drug-treatment. To study the modulating effect of anthracyclines on the "secrotome" and on protein surface display by a proteomic approach will also be very interesting.
3. The finding that rpP2 is dephosphorylated early in apoptosis claims for further study of the effect of dephosphorylation on translation. Constructs with mutations in the phosphorylation-sites of rpP2 have been generated and to see how these influence translation will be interesting. The possibility of using rpP2 as a target for intelligent drug design will have to be explored.
4. The finding that a protein synthesis inhibitor enhanced anthracycline-induced apoptosis in AML animal models suggests that this could be an interesting combination for an anthracycline-based combination regime. The combination regime should be tested on other animals as well such as e.g. mini-pigs and dogs. While CHX is a global inhibitor of protein synthesis and probably not suitable for use on people, a search/screen for alternative protein synthesis inhibitors that are less toxic and more specific in their action are warranted.

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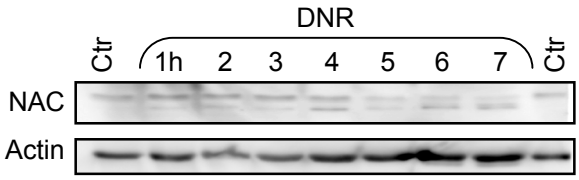
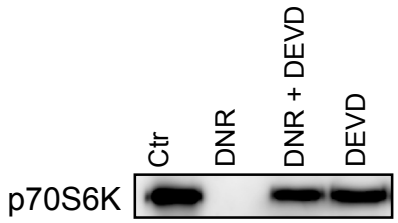
Supplementary data

Supplementary Figure 1



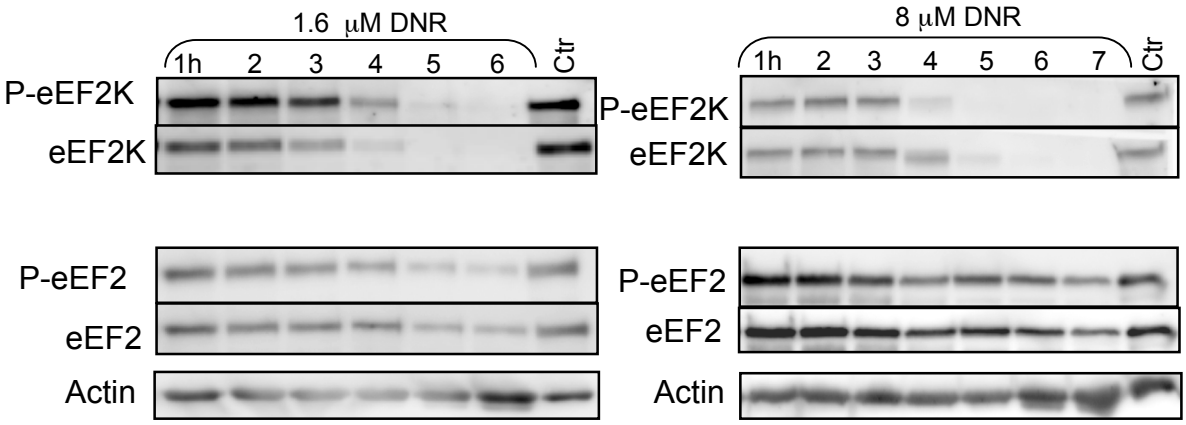
NAC is cleaved in DNR-treated cells. HL60 cells were treated with 8 μM DNR for the times indicated and cell extracts immunoblotted and probed for NAC.

Supplementary Figure 2



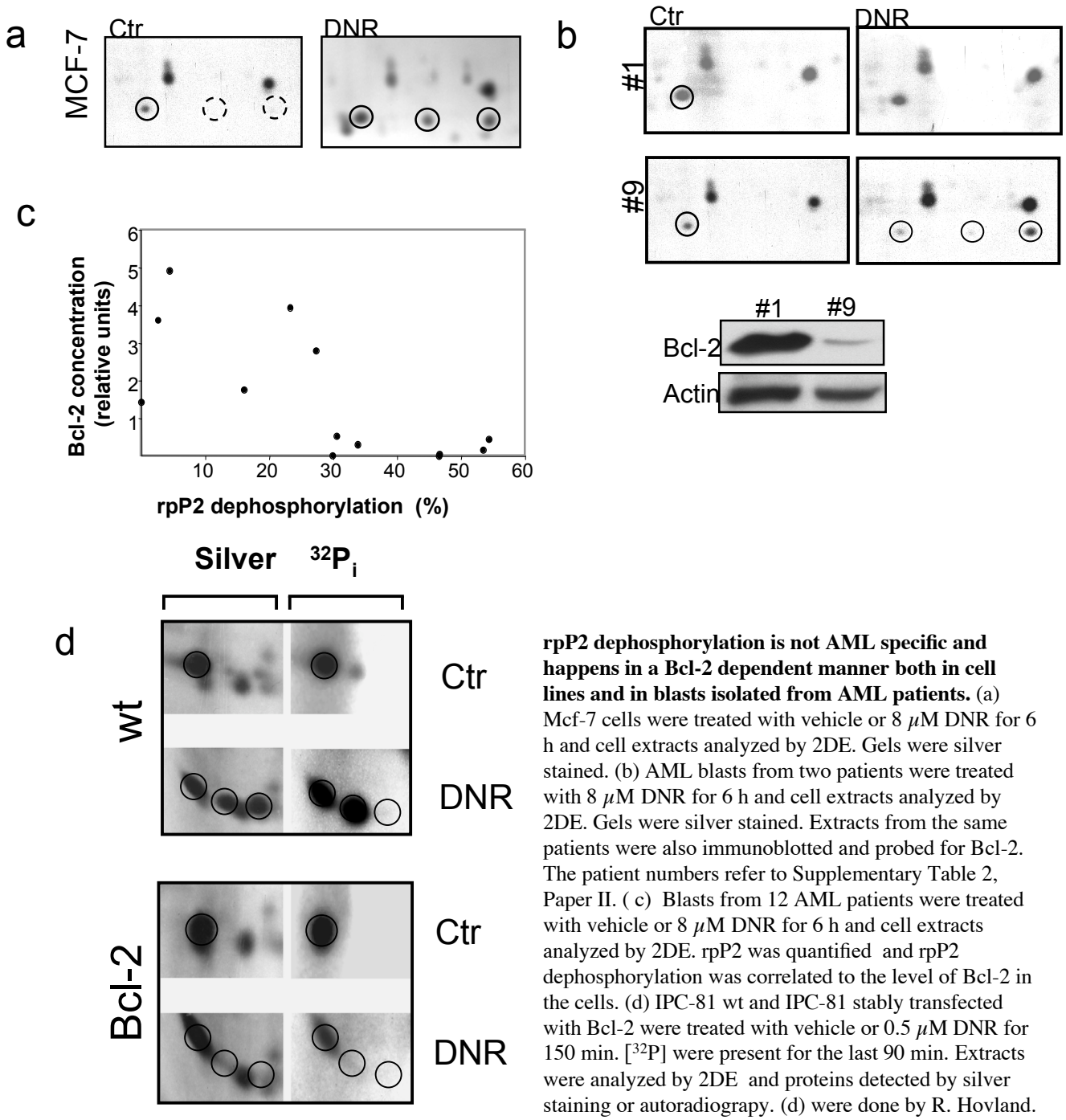
p70S6K is degraded in a caspase-mediated way. HL60 cells were treated with 8 μM DNR for 6 h in the presence or absence of the caspase 3/7 inhibitor DEVD (50 μM). Cell extracts were immunoblotted and probed for p70S6K.

Supplementary Figure 3



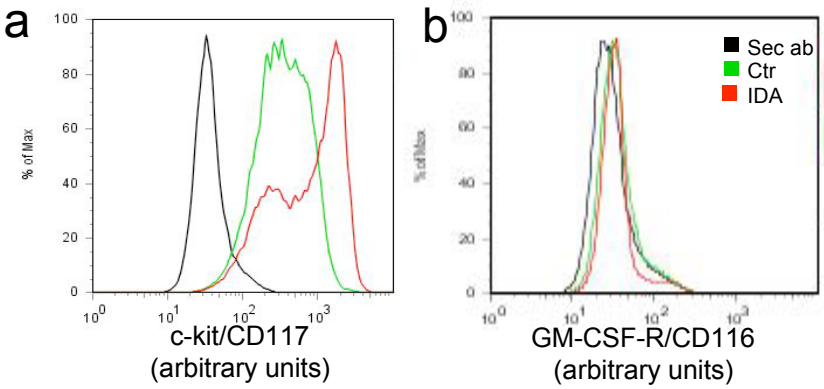
Effect of DNR on eEF2 and eEF2K. HL60 cells were treated with 1.6 or 8 μM DNR for the periods indicated, extracts immunoblotted, and probed for P-eEF2K, eEF2K, P-eEF2 and eEF2.

Supplementary Figure 4



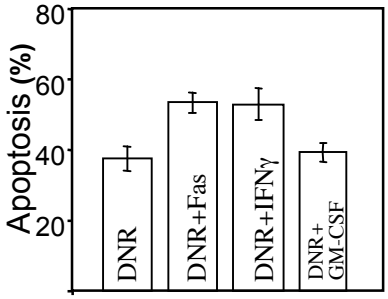
rpP2 dephosphorylation is not AML specific and happens in a Bcl-2 dependent manner both in cell lines and in blasts isolated from AML patients. (a) Mcf-7 cells were treated with vehicle or 8 μM DNR for 6 h and cell extracts analyzed by 2DE. Gels were silver stained. (b) AML blasts from two patients were treated with 8 μM DNR for 6 h and cell extracts analyzed by 2DE. Gels were silver stained. Extracts from the same patients were also immunoblotted and probed for Bcl-2. The patient numbers refer to Supplementary Table 2, Paper II. (c) Blasts from 12 AML patients were treated with vehicle or 8 μM DNR for 6 h and cell extracts analyzed by 2DE. rpP2 was quantified and rpP2 dephosphorylation was correlated to the level of Bcl-2 in the cells. (d) IPC-81 wt and IPC-81 stably transfected with Bcl-2 were treated with vehicle or 0.5 μM DNR for 150 min. [^{32}P] were present for the last 90 min. Extracts were analyzed by 2DE and proteins detected by silver staining or autoradiograpy. (d) were done by R. Hovland.

Supplementary Figure 5



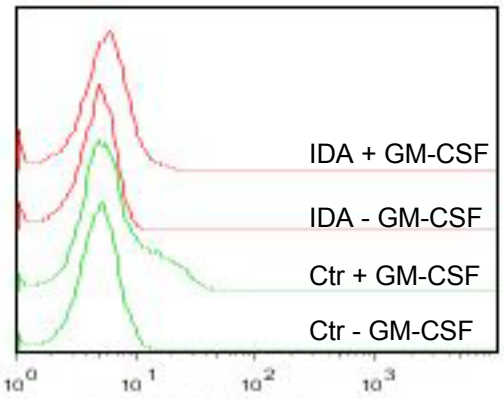
Surface receptor accumulation after IDA treatment. (a) NB4 cells were treated with vehicle or IDA (720 nM, 5 h) and analyzed for surface expression of c-kit/CD117. (b) HL60 cells were treated with vehicle or IDA (720 nM, 5 h) and analyzed for GM-CSF-R/CD116.

Supplementary Figure 6



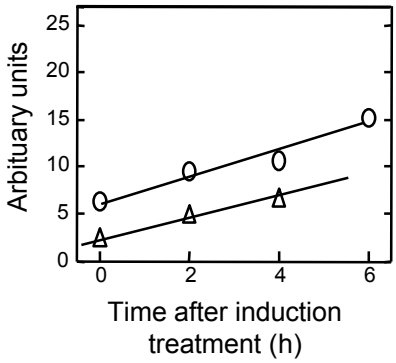
Modulation of DNR-induced apoptosis. HL60 cells were treated with vehicle or DNR (1.6 µM, 4 h) in the presence or absence of monoclonal antibody towards Fas-R (1 µg/ml), IFNγ (20 ng/ml) or GM-CSF (20 ng/ml), and apoptosis was scored. Data represents ±SEM of 3 experiments.

Supplementary Figure 7



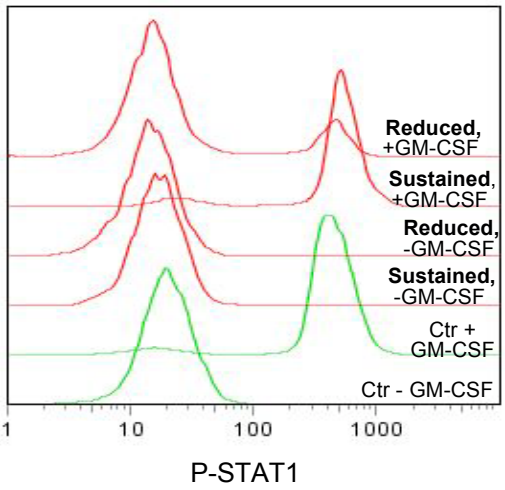
Abrogation of STAT5 signaling after IDA treatment. HL60 cells were treated with vehicle or IDA (720 nM, 5 h) and stimulated with GM-CSF (20 ng/ml) the last 15 min. Alexis488-conjugated transferrin was present the last 10 min, and P-STAT5 were analyzed by FACS. For IDA treated cells, the two populations differing in transferrin endocytosis (reduced endocytosis, shown, and sustained endocytosis, not shown and identical to Ctr) were separately analyzed for P-STAT5.

Supplementary Figure 8



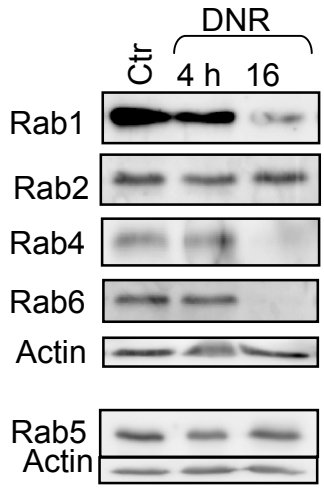
Flt3 accumulates on the surface of AML blasts during induction treatment. AML blasts from two patients (O, #7 and Δ, #6) were collected before and 2, 4 and 6 h after start of IDA+AraC treatment. Isolated blasts were assayed for surface expression of Flt3. See Paper III for patient information and methodology.

Supplementary Figure 9



Abrogation of STAT1 signaling after IDA treatment. HL60 cells were treated with vehicle (green) or IDA (red, 720 nM, 5 h) and stimulated with GM-CSF (20 ng/ml) the last 15 min. Alexis488-conjugated transferrin were present the last 10 min, and P-STAT1 were analyzed by FACS. For IDA treated cells, the two populations differing in transferrin endocytosis (reduced Tf endocytosis and sustained Tf endocytosis) were separately analyzed for P-STAT1.

Supplementary Figure 10



Effect of DNR on the family of Rab GTPases. HL60 cells were treated with vehicle or 8 μM DNR for 4 or 16 h and cell extracts immunoblotted and probed for members of the Rab family.

Supplementary Table 1. Proteins related to RNA binding and protein synthesis found by COFRADIC N-terminal analysis to be altered in DNR+CHX treated NB4 cells.

A: Internally located alpha-N-acetylated peptides generated after aspartic acid specific cleavage.

Protein Description	Accession number	Cleavage Site	Functions
H/ACA ribonucleoprotein complex subunit 4	O60832	EYVD↓	Pseudouridine synthase involved in rRNA posttranscriptional modification [1]
Heterogeneous nuclear ribonucleoprotein A0	Q13151	HAVD↓	mRNA translation regulation [2,3]
Heterogeneous nuclear ribonucleoprotein U	Q00839	PAGD↓	Nuclear retention, packing and processing of RNA [4], part of IMP1 RNP Granules [5], scaffold mRNA-protein interactions [6], scaffold DNA-interacting protein [7]. Cleavage results in detachment of protein from nuclear scaffold, could contribute to nuclear structure collapse, cleavage does not affect function in RNA metabolism [8,9]
Nascent polypeptide-associated complex subunit alpha	Q13765	EEQD↓	Binds nascent polypeptide chains and inhibits ER targeting [10], regulates FADD function [11], transcriptional co-activator [12]
Polyadenylate-binding protein 2	Q86U42	VEGD↓	mRNA transport [13], stimulates addition [14] and controls size of the poly(A)-tail [15], translational regulation [16]
RNA-binding protein 39	Q14498	ERTD↓	Co-localizes with core spliceosomal proteins, possibly a splice factor [17]
Splicing factor U2AF 65 kDa subunit	P26368	MTPD↓	Initiation of spliceosome assembly, binds directly to polypyrimidine tracts in the 3`-UTR [18]
TAR DNA-binding protein 43	Q13148	DETD↓	Regulates splicing of the cyctic fibrosis transmembrane conductance regulator (CFTR) gene [19]
U6 snRNA-associated Sm-like protein LSm3	P62310	DDVD↓	mRNA decapping [20] and pre-mRNA splicing [21]

B: N-terminal peptides with decreased expression.

Protein Description	Accession number	Fold down-regulation	Function
Elongation factor 2 kinase, eEF2K	O00418	>10	Ca^{2+}/calmodulin-dependent kinase, eEF2 only known substrate [22,23]
Eukaryotic translation initiation factor 4H, eIF4H	Q15056	>10	Initiation of protein synthesis [24], modulates eIF4A helicase activity [25].
Lysyl-tRNA synthetase	Q15046	>10	Ligates lysine to tRNA [26,27], interacts with eEF-1 [28]
60S ribosomal protein L13a	P40429	5.9	Structural part of 60S ribosomal subunit, transcript-specific translational control [29,30]
Heterogeneous nuclear ribonucleoproteins C1/C2	P07910	4.4	Translational modifier of c-myc [31], stimulates IRES-mediated translation [32], mRNA transport out of nucleus [31,33]
		2.9	

Asparaginyl-tRNA synthetase, cytoplasmic	O43776	4.2	Ligates asparagine to tRNA [26,34]
40S ribosomal protein S30	P62861	2.5	Structural part of the 40S ribosomal subunit [35]
60S ribosomal protein L30	P62888	2.5	Structural part of 60S ribosomal subunit [36]
U6 snRNA-associated Sm-like protein LSm3	P62310	2.5	mRNA decapping [20] and pre-mRNA splicing [21]
Elongation factor 2, eEF2	P13639	2.4	Ribosomal translocation during elongation, GTPase [23]
Eukaryotic translation initiation factor 1A,	O14602	2.3	Promotes 43S complex formation, ensures fidelity of AUG-codon selection [37,38]
Phenylalanyl-tRNA synthetase alpha chain	Q9Y285	2.3	Ligates phenylalanine to tRNA [26], interacts with EF-1 [28]
40S ribosomal protein S3a	P61247	2.2	Structural part of 40S ribosomal subunit [35], interacts directly with mRNA [39], inhibits PARP [40]
FK506-binding protein 1A	P62942	2.1	Promotes binding of rapamycin to mTOR [41]

A subset of internally located alpha-N-acetylated peptides (A) and peptides with decreased (B) expression after induction of apoptosis in NB4 cells with DNR (1.6 µM) and CHX (3.6 µM) for 8 h (80 % apoptosis) are listed. Only peptides with corresponding parent proteins with functions related to mRNA binding/processing and protein synthesis are shown. The complete list of peptides is given in Paper II. Peptide identification was done using "UniProt_SwissProt and UniProt_TrEMBL" databases. The parent proteins are referred to by description and UnitProt database accession number. The peptides B indirectly indicate proteolytic processing of their parent proteins.

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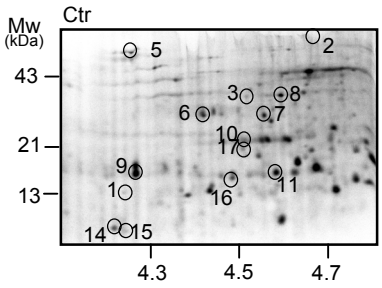
Supplementary Table 2

Effect of DNR on the relative 35S-methionine incorporation of spesific proteins based on *in vitro* mRNA translation.

Spot number	Protein abbreviation	Fold increase (DNR/Ctr)
1	rpP2	1.93
2	P4HB	~1
3	PCNA	0.27
4	cutA	n.d
5	CALR	0.55
6	eEF1Ba	0.86
7	PSMA5	0.50
8	CTM	1.39
9	MLC	1.12
10	n.i	0.91
11	n.i	0.81
12	n.i	n.d
13	n.i	n.d
14	n.i	0.5
15	n.i	0.95
16	n.i	1.1



Cells were treated with vehicle or 8 μM DNR for 4.5 hours and proteins translated from RNA isolated from vehicle- (Ctr, shown) or DNR-treated cells in the presence of [^{35}S]-methionine were analyzed by 2DE (pI 4-5). Protein spots were analyzed for relative labeling intensity. The ratio between relative spot intensity in DNR- and vehicle-treated cells after *in vitro* translation was calculated. Protein abbreviations are as in Table 1, Paper II. n.i.: not identified, n.d.: not determined. The data are average from 3 separate experiments. The numbers in the table refers to the proteins spots in Figure 4B, Paper II and the autoradiogram of the 2DE gel shown here.

Supplementary Table 3. Internally located alpha-N-acetylated peptides generated after IDA/bortezomib-induced apoptosis

Protein Description	Accession number	Start	End	Identified Peptide	Site
VAMP-8, endobrevin	Q9WUF4	21	32	Ac-GVKNIM<Mox>TQNVER	SEVE_
hnRNP-Q, synaptotagmin-binding protein	Q7TP47	316	323	Ac-AKPPDQKR	EIVF_
Spectrin alpha chain	P16086	38	45	NH2-Q<Pyr>KLEDSYR	TLRR_
		1206	1214	Ac-N<Dam>SIKELNER	VATF_
SNARE-associated protein Snapin	P60192	83	89	Ac-KKLLN<Dam>AR	DPYV _
SNAP-29	Q9Z2P6	75	83	NH2-VASSEELVR	EKIG _
Syntaxin-binding protein 5 (Tomosyn-1)	Q9WU70	1008	1016	Ac-LVSPTEIQR	QALY_
Intersectin-1, regulator of endocytosis 1	Q9WVE9	361	367	Ac-N<Dam>LELEKR	EFRG_
Unc-13 homolog A (Munc13-1)	Q62768	31	39	Ac-VKSTTIAVR	KVQN _
Myosin-9	Q62812	1629	1641	NH2-ANKNREEAIKQ<Dam>LR	HIDT_
Spectrin beta chain, brain 2	Q9QWN8	1042	1051	Ac-EVQ<Dam>TGWEDLR	TRLG_
Tektin-4	Q6AXV2	130	137	Ac-Q<Dam>MM<Mox>AQKLR	SETD_
Vacuolar protein sorting-associated protein 33A (r-vps33a)	Q63615	10	20	NH2-VN<Dam>LNVLREAVR	SYGR_
Paxillin	Q66H76	149	157	Ac-LLELNAVQR	LDRL_
Ezrin	P31977	299	309	NH2-IEVQ<Dam>QMKAQAR	KPDT_

A subset of proteins detected by N-terminal COFRADIC-analysis of IPC-81 cells treated with IDA (100 nM) and bortezomib (30 nM) for 6 h. Only peptides with corresponding parent proteins with functions related to cellular vesicle trafficking are shown. Amino acids preceding the identified peptide are indicated (site). The parent proteins are referred to by description and SwissProt accession number. Ac- indicates acetylation, <Dam> deamidation, <Mox> methionine oxidation and <Pyr> cyclic pyro-glutamate peptide.

Errata

Figure 7a page 45: eIF4A should be eIF1A.
Figure 7b, page 46, mRNA should be single stranded.

Page 100, Supplementary Table 2. Spot number 2 should be called PDI/P4HB.

PaperII
Page 40, Figure 5D. Heading should be ”Internally located alpha-N-acetylated peptides generated by cleavage after aspartic acid in DNR/CHX-treated cells”.

Paper I

Caspase-dependent, geldanamycin-enhanced cleavage of co-chaperone p23 in leukemic apoptosis.

Gro Gausdal, Bjørn Tore Gjertsen, Kari Espolin Fladmark, Hans Demol, Joël Vandekerckhove and Stein Ove Døskeland

Leukemia (2004) 18, 1989–1996

Leukemia 18, Gausdal, G.; Gjertsen, B. T.; Fladmark, K. E.; Demol, H.; Vandekerckhove, J. and S. O. Døskeland, Caspase-dependent, geldanamycin-enhanced cleavage of co-chaperone p23 in leukemic apoptosis, pp. 1989–1996. Copyright 2004 Nature Publishing Group. All rights reserved. Abstract only. Full-text not available due to publisher restrictions. The published version is available here: http://dx.doi.org/10.1038/sj.leu.2403508

Caspase-dependent, geldanamycin-enhanced cleavage of co-chaperone p23 in leukemic Apoptosis

G Gausdal, BT Gjertsen, KE Fladmark, H Demol, J Vandekerckhove and S-O Døskeland

Co-chaperone p23 is a component of the heat-shock protein (Hsp)90 multiprotein-complex and is an important modulator of Hsp90 activity. Hsp90 client proteins involved in oncogenic survival signaling are frequently mutated in leukemia, and the integrity of the Hsp90 complex could therefore be important for leukemic cell survival. We demonstrate here that p23 is cleaved to a stable 17 kDa fragment in leukemic cell lines treated with commonly used chemotherapeutic drugs. The cleavage of p23 paralleled the activation of procaspase-7 and -3 and was suppressed by the caspase-3/-7 inhibitor DEVD-FMK. In vitro translated 35S-p23 (in reticulocyte lysate) was cleaved at D142 and D145 by caspase-7 and -3. Cleavage of p23 occurred in caspase-3-deficient MCF-7 cells, suggesting a role for caspase-7 in intact cells. The Hsp90 inhibitor geldanamycin enhanced caspase-dependent p23 cleavage both in vitro and in intact cells. Geldanamycin also enhanced anthracycline-induced caspase activation and apoptosis. We conclude that p23 is a prominent target in leukemic cell apoptosis. Geldanamycin enhanced p23 cleavage both by rendering p23 more susceptible to caspases and by enhancing chemotherapy-induced caspase activation. These findings underscore the importance of the Hsp90-complex in antileukemic treatment, and suggest that p23 may have a role in survival signaling.

Keywords: AML; anthracyclines; co-chaperone p23; geldanamycin; caspase-7

Paper II

Abolition of stress-induced protein synthesis sensitizes leukemia cells to anthracycline-induced death.

Gro Gausdal, Bjørn Tore Gjertsen, Emmet McCormack, Petra Van Damme, Randi Hovland, Camilla Krakstad, Øystein Bruserud, Kris Gevaert, Joël Vandekerckhove, and Stein Ove Døskeland

Manuscript, in revision at Blood

Abolition of stress-induced protein synthesis sensitizes leukemia cells to anthracycline-induced death

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Scientific heading: Neoplasia

Running title: Translational Control by Anthracyclines

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Abstract

Anthracycline action has been thought to involve the neosynthesis of pro-apoptotic gene products, and therefore depend on protein synthesis for optimal effect. We found that inhibition of general, but not rapamycin-sensitive (cap-dependent), protein synthesis in the pre-apoptotic period enhanced anthracycline-induced AML cell death, both *in vitro* and in several animal AML models. Pre-apoptotic anthracycline-exposed AML cells had altered translational specificity, with enhanced synthesis of a subset of proteins, including ER chaperones. The altered translational specificity could be explained by perturbation (protein degradation, truncation or dephosphorylation) of the cap-dependent translation initiation machinery and of proteins controlling translation of specific mRNAs. We propose that judiciously timed inhibition of cap-independent translation is considered for combination therapy with anthracyclines in AML.

Paper III

Clathrin light chain is targeted by anthracyclines and results in blocked endocytosis and attenuated receptor mediated survival signaling in leukemia

Gro Gausdal, Jørn Skavland, Kris Gevaert, Joël Vandekerckhove, Stein Ove Døskeland and Bjørn Tore Gjertsen

Manuscript

Clathrin light chain is targeted by anthracyclines and results in blocked endocytosis and attenuated receptor mediated survival signaling in leukemia

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Short title: Anthracycline targets clathrin-dependent endocytosis in AML.

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ABSTRACT

Anthracyclines are among the most efficient chemotherapeutics against acute myelogenous leukemia (AML), but their use is limited by toxicities and lack of persisting remissions. Understanding the molecular mechanisms behind cell death induced by the polypharmacologic anthracyclines may facilitate development of more efficient and less toxic treatment. Using two-dimensional polyacrylamide gel electrophoresis and an immunoblot screen, we found that clathrin-dependent endocytosis was targeted through degradation of numerous proteins involved in clathrin-mediated endocytosis. Clathrin light chain (CLC) was the only protein to be degraded in the pre-apoptotic phase and CLC was also targeted in patients receiving induction treatment. Early after drug-treatment, transferrin endocytosis was abolished and receptors involved in death and life-signaling accumulated on the cell surface. This was due to caspase and proteasomal degradation of proteins, proposed by restored endocytosis by inhibition of caspase or proteasomal activity and through mutational disruption of the destruction box in CLC. Attenuated clathrin dependent endocytosis was accompanied by compromised survival signaling through growth factor receptors. Several lines of evidence suggest that CLC plays a prominent role in pre-apoptotic targeting of the endocytic machinery. We hypothesize that the anthracycline targeting of endocytosis and growth factor signaling contributes to their successful cancer cell debulkment.

INTRODUCTION

The anthracyclines, notably daunorubicin (DNR) and idarubicin (IDA), have been the backbone in AML therapy for decades [1,2]. Even though direct effects of the anthracyclines such as DNA intercalation, topoisomerase II inhibition and generation of oxidizing radicals contribute to apoptosis induction, the actions of anthracyclines in cell death induction are incompletely understood [3,4]. Anthracyclines are hampered with adverse effects, particularly cardiotoxicity, and many patients experiences disease relapse after treatment. New insight into the therapeutic mechanisms of anthracyclines could therefore be beneficial to enhance the therapy and avoid toxicities.

Expression of constitutively activated receptor tyrosine kinases is proposed to induce drug resistance, and inhibition of such receptors enhances the cellular effect of anthracyclines [5-8]. Internalization of cytokine receptors through clathrin-dependent endocytosis may be a prerequisite for signaling and downstream activation of signal transducers and activators of transcription (STATs) [9].

The clathrin triskelion is a triskelion composed of three heavy chains (CHC) and three light chains (CLC) [10]. Whereas the heavy chains of clathrin form the structural backbone of the triskelion, the tree smaller light chains have a less clear role. However, they are thought to regulate the formation or disassembly of the clathrin coats. While studies have shown that CLC is dispensible for clathrin triskelions assembly *in vitro* [11], CLC is important for assembly of triskelions onto intracellular membranes in the amoeba *Dictyostelium* [12], in yeast [13,14] and in mammalians [15].

We analysed cellular protein modifications induced by anthracyclines by 2-dimensional gel electrophoresis and immunoblotting to elucidate the early apoptotic responses to

anthracyclines. We found pre-apoptotic degradation of CLC, attenuated clathrin dependent endocytosis and decoupled survival signaling from growth factor receptors. This elucidates a central role for clathrin light chain in receptor signaling and propose a new therapeutic target in future therapy

MATERIALS AND METHODS

Reagents

RPMI medium, horse serum (HS) and fetal bovine serum (FBS) were from Gibco (Grant Island, NY, USA). Daunorubicin (DNR) and cycloheximide (CHX) were from Sigma (St. Louis, MO), and idarubicin (IDA) was from Pharmacia AB (Stockholm, Sweden). zVAD-fmk and MG132 were from Alexis Corp. (Läufelfingen, Switzerland). Anti-Fas IgM was from Upstate, IFNγ from Peprotech Inc. (Rocky Hill, NJ). Transferrin conjugated with Alexa Fluor488 or 647 and ProLong antifade kit were from Molecular Probes (Eugene, OR). Monoclonal antibodies against Fas-R/CD95 (FITC-conjugated) was from BD-Bioscience (Oslo, Norway), TfR/CD71 from Diatec (Oslo, Norway), IFNγ-R/CD119 from Santa Cruz Biotechnology, and isotype control (mouse IgG_1, FITC/PE conjugated) were from Immunotech (Marseille, France). Annexin V-FITC was from Nexins Research (Kattendijke, Netherlands).

Cell lines and patient material

The HL60 cell line was from GCMCC (www.dsmz.de) and the NB4 cell line from Dr. M Lanotte (INSERUM U-496, Hôp. St Louis, Paris, France). They were cultured in RPMI medium with 10% FBS and L-glutamine (2 mM) and kept in logaritmic growth until studied at about $0.5x10^6$ cells/ml. The IPC-81 rat promyelocytic leukemia cell line was

cultured as described [16]. Primary AML blasts for *in vivo* analysis were collected before and after start of the chemotherapy induction course. Blood were collected by cubital vein puncture while chemotherapeutics were infused through a central venous catheter. Cells were separated by Ficoll gradient centrifugation [17], and freshly isolated cells were assayed or washed once in 9% saline and precipitated in 7% trichloroacetic acid (TCA) for 2DE analysis. Primary AML blasts for *in vitro* analysis were collected from patients with >90% blasts in peripheral blood, separated by Ficoll gradient centrifugation and cultured in StemSpan medium (StemCell Technologies, Vancouver, Canada) [18]. Informed consent was obtained from all patients, and detailed information about patient material is given in Supplementary Table 1.

Immunoblotting, 2DE-gel electrophoresis and MALDI-TOF-MS analysis

Immunoblotting was as described [19]. Antibodies against CLC, procaspase-3, Rab5, Rab7 and actin were from Santa Cruz Biotech. Antibodies against CHC, AP2-α, dynamin, AP180, EEA1, Eps15 and Rab4 were from BD Biosciences. CIN85 antibody was from Upstate. Antibodies against Rab1, Rab2, Rab6 and caveolin were provided by Dr. J Saraste, University of Bergen, Norway.

[^{35}S]-methionine metabolic labeling of cells, 2DE-gel electrophoresis, staining of gels, picking of spots and MALDI-TOF-MS analysis was essentially as described [19 and Gausdal et al., Paper II].

Assessment of apoptosis

Cells were fixed in phosphate-buffered saline with 2% formaldehyde and screened for apoptosis using differential interference contrast microscopy to visualize surface budding and fluorescence microscopy for chromatin condensation. The chromatin pattern in cells incubated with the autofluorescent anthracyclines was identical to that observed with the DNA specific dyes bisbenzimide H33342 and DAPI [20]. Apoptosis was also scored by flow cytometric analysis of forward and side light scattering [21] and by AnnexinV staining.

Transferrin endocytosis, surface receptors and intracellular phosphoproteins

To study transferrin endcytosis, cells were exposed to experimental drugs and fluorescently conjugated transferrin was preset at 100 μg/ml during the 10 last minutes of treatment. Cells were washed once in 20 vol of 0.9% NaCl at 4°C and then fixed in 1 vol 4% paraformaldehyde. For microscopy, the cells were mounted with ProLong antifade kit and analyzed using a BioRad MRC1024 scanning confocal system.

Determination of Fas-R/CD95, IFNγ-R/CD119, TfR/CD71 was performed following the suppliers' instructions. Cells exposed to experimental drugs were washed ones in 20 vol of 0.9% NaCl at 4°C and then fixed in one vol 1.5% paraformaldehyd prior to staining with respective antibody. Alexa647 $F(ab')_2$ was used as secondary antibody for detection by flow cytometry.

Preparation of cells for intracellular phospho-protein analysis were performed as previously described [22] and following the supplier's instruction for antibody concentration and time of incubation. Anti P-STAT5 (pY694) were from BD

Biosciences, Norway. Samples were analyzed in a Becton Dickinson FACSCalibur flow cytometer.

CLC constructs, mutagenesis and virus transfection

CLC a-chain cDNA was subcloned into pEGFP-C3 (BD Biosciences), and mutations in CLC (R205A and L208A) was generated by overlapping PCR primer extension (primers 5´-gtctcccgcatggcctcagtcgccatctccctcaag-3´ and 5´ - cttgagggagatggcgactgaggccatgcgggagac-3`). The wildtype and mutated CLC was sub-cloned into a retroviral vector (CRU5-IRES-GFP) allowing CLC and GFP to be translated form a single bicistronic mRNA. Enzyme digestion and DNA sequencing verified all constructs.

Virus production was performed as described [23]. Spin infection of NB4 cells was performed by centrifugation at 1200 x g for 90 min (32°C) followed by incubation for 48 h. About 10% of the cells showed GFP fluorescence, and positive cells were isolated on a FACS Aria (BD).

RESULTS

Anthracyclines induce modifications in clathrin light chain (CLC) and surface receptor endocytosis both in cell lines and in blasts from patients under induction chemotherapy.

Cell extracts from HL60 cells treated with vehicle or DNR were subjected to 2DE analysis. One spot (Mw 28 kDa, pI 4.4) absent in extract from DNR-treated cells was identified by MS as clathrin light chain (CLC) (Figure 1A). The DNR-induced

downregulation of CLC was due to degradation and not decreased translational efficiency or CLC-mRNA levels, since CLC was synthesized *in vitro* from mRNA isolated from both vehicle and DNR-treated cells (Figure 1B) and since CLC was degraded in cells prelabeled with [^{35}S]-methionine and chased in the presence of cold methionine and DNR (Figure 1C).

CLC is involved in stabilization of the clathrin lattice, but the exact role of CLC in clathrin-dependent endocytosis is not known. To determine whether clathrin-mediated endocytosis was affected by DNR treatment, we examined uptake of transferrin after drug-treatment since receptor-mediated transferrin uptake is dependent on endocytosis via clathrin coated vesicles [24]. Scanning confocal microscopy revealed that the amount of internalized fluorescently labeled transferrin was markedly decreased in cells already after 2 h of incubation with DNR, and most interestingly, before the cells showed morphological signs of apoptosis such as nuclear chromatin condensation (Figure 1D). When the effect of IDA on transferrin uptake was determined by FACS analysis, the cells divided into two populations, one with decreased uptake of transferrin and one with uptake that was identical to vehicle treated cells (Figure 1E and not shown). Neither of the two cell populations showed morphological signs of apoptosis judged by forward and side scatter, indicating that clathrin-mediated endocytosis is modified in the early phases of anthracycline-induced apoptosis.

The decrease in internalization of transferrin could be due either to degradation of the receptor itself or to inhibited endocytosis of the receptor. We therefore studied the effect of IDA on the surface expression of transferrin receptor (TfR) and found that the population of cells with reduced transferrin uptake (Figure 1E) showed accumulated TfR

on the cell surface (Figure 1F). This indicates that the reduced transferrin uptake was due to decreased TfR endocytosis.

Clathrin-mediated endocytosis is a major mechanism for the cell to transmit signals from the exterior to the interior of the cell [25]. Consequently, interfering with this process could be important for how a cancer cell respond to chemotherapy and could have clinical relevance. We therefore isolated blasts from patients with diagnosed AML and treated them with DNR *in vitro*. 2DE analysis showed degradation of CLC in AML blasts (Figure 2A). AML blasts were also isolated from 3 patients before and after the onset of induction treatment, and 2DE analysis of cell extracts revealed that CLC was partially or completely degraded in patients receiving induction chemotherapy (Figure 2B). In the body, cells with morphological signs of apoptosis are removed from the circulation [26]. Hence, the degradation of CLC seen in blasts isolated from patients receiving chemotherapy indicates that this takes place in the pre-apoptotic phase.

To study if endocytosis was affected during the course of induction treatment, primary AML blasts from 2 patients collected before and after start of treatment were analyzed by FACS for internalization of transferrin and for surface expression of Fas receptor (Fas-R). Within 6 h after start of induction treatment, the uptake of transferrin was reduced (Figure 2C) and Fas-R accumulated on the surface of the AML blasts (Figure 2D). Nuclear chromatin staining (not shown) and FACS analysis of AnnexinV labeled cells showed that the amount of Annexin-positive cells did not increase substantially during the treatment (Figure 2E). We suggest, based on degradation of CLC, decreased transferrin uptake and accumulation of receptors on the surface on AML cells harvested

from patients under chemotherapy, that clathrin-mediated endocytosis is pre-apoptotically targeted in anthracycline treatment *in vivo*.

Anthracyclines induce caspase- and proteasome-mediated degradation of proteins involved in clathrin-mediated endocytosis

Clathrin-mediated endocytosis is reported sustained in the absence of CLC [27]. Therefore, since anthracycline-treated pre-apoptotic cells demonstrated both compromised transferrin endocytosis and CLC degradation, we questioned if other components of the endocytic machinery were targeted during apoptosis. An immunoblot screen including several proteins involved in clathrin-mediated endocytosis, revealed that a number of proteins known to be structurally or regulatory involved in clathrin-mediated endocytosis were targeted for degradation in anthracycline-induced apoptosis. Both clathrin heavy chain (CHC) and proteins involved in assembly of the clathrin triskelion, namely AP180, AP2-α and Eps15 were degraded or cleaved in DNR treated cells (Figure 3A). Also EEA1, involved in fusion of early endosomes, dynamin, the GTPase responsible for release of clathrin coated vesicles from the membrane and CIN85, a regulator of ubiquitin ligases involved in receptor cycling, were modified upon anthracycline treatment (Figure 3A). Structurally analogous adaptors that mediate clathrin-dependent vesicular transport between the trans-Golgi network and the endosomes (AP1-γ, AP3-δ and AP3-β) were not modified during DNR-treatment (data not shown).

We investigated cleavage and degradation kinetics of these proteins to determine if CLC was an early proteins affected by anthracycline treatment. HL60 cells were treated with DNR for 1 to 6 h and cell extracts analyzed by immunoblotting. The majority of the

proteins show a cleavage-pattern that coincides with activation of procaspase-3. This accounts for Eps15, EEA1, AP2-α, CIN85 and dynamin (Figure 3B). Notably, the activation of procaspase-3 occurs at the same time as the cells start to show morphological signs of apoptosis (Figure 3C) indicating that the cleavage of these proteins are probably related to the execution phase of apoptosis. The completeness of cleavage varies between the proteins. While EEA1, Eps15 and AP2-α show a more or less complete cleavage/degradation, CIN85 and in particular dynamin, are only partially cleaved. The only protein that is degraded before activation of procaspase-3, is CLC (Figure 3B). This supports indications that CLC is targeted pre-apoptotically (Figure 2B) and we hypothesized that degradation of CLC is related to apoptosis induction.

In addition to clathrin-mediated endocytosis, the cell also has other pathways for internalizing surface receptors and extracellular substances. Clathrin-independent endocytosis has been considered to occur through cholesterol- and sphingolipid-enriched membrane domains such as lipid rafts and caveolae [28]. Caveolin, a cholesterol-binding protein associated to caveola was not degraded in DNR-induced apoptosis (Figure 3B).

Anthracyclines are well known to induce caspase activation [29]. As targeting of most of the proteins coincided with activation of procaspase-3, we wondered whether the protein modifications were due to caspase-mediated cleavage. Cells were treated with anthracyclines and the broad range caspase inhibitor zVAD and cell extracts analyzed by immunoblotting. While the drug-induced effect on Eps15, CIN85, EEA1 and dynamin could be completely inhibited by the presence of zVAD, CLC and AP2-α were only partially rescued even in the presence of high doses of the caspase inhibitor (Figure 4A) indicating that the degradation of CLC and AP2-α were not solely due to caspases. An

alternative way of degrading proteins is through the proteasome. The presence of the proteasome inhibitor MG132 totally blocked the degradation of AP2-α and partially blocked degradation of CLC (Figure 4B).

Having shown that several essential proteins involved in clathrin-mediated endocytosis were targets for caspase- or proteasome-mediated degradation, we wanted to see whether we could restore the decrease in transferrin endocytosis by inhibiting their degradation. By co-treating cells with DNR and zVAD or MG132, we were able to restore transferrin endocytosis to the level seen in vehicle-treated cells (Figure 4C). We conclude that anthracyclines induce degradation and cleavage of several proteins involved in clathrin-mediated endocytosis and that this results in a downregulation of clathrin-mediated transferrin endocytosis.

Cytokine receptor expression and signaling are modified in anthracycline-treated cells.

Receptor internalization by clathrin-mediated endocytosis plays an essential role in surface receptor signaling [25]. Several receptors known to modulate the effect of apoptosis-inducing drugs, including Fas-R and interferon gamma receptor (IFNγ-R) are internalized after ligand interaction. However, the role of internalization for signaling is still elusive [9,30-32]. Our Fas analysis of blasts isolated from patients receiving chemotherapy indicated that Fas accumulated on the cell surface, and we therefore studied receptors and their signaling more in detail in cell lines. Treatment of NB4 and HL60 cells (not shown) with IDA induced a surface accumulation of both Fas-R (Figure 5A) and IFNγ-R (Figure 5B). The accumulation was not due to increased synthesis of

receptors as the protein synthesis inhibitor cycloheximide (CHX) did not prevent accumulation (Figure 5A,B).

STAT5 phosphorylation is involved in transferring cytokine receptor signaling into an anti-apoptotic response. NB4 (not shown) and HL60 show STAT5 phosphorylation upon stimulation with IFNγ (Figure 5C). This phosphorylation was inhibited upon IDA treatment in cells with attenuated transferrin uptake, while cells with normal transferrin uptake displayed normal STAT5 response (Figure 5C and not shown). Both cells with high and low transferrin uptake demonstrated forward and side scatter signal identical to vehicle treated cells.

A possible modulation of anthracycline-induced apoptosis through these receptors was next examined, and we found that IFNγ and monoclonal antibodies against Fas-R markedly enhanced DNR-induced apoptosis (Figure 5D).

We conclude that surface receptors with the ability to modulate cellular response to drug exposure accumulate during anthracycline-induced apoptosis and that anthracycline-induced inhibition of STAT5 activation correlates with a reduction in clathrin-mediated endocytosis.

Destruction-box motif involved in anthracycline-induced CLC degradation and endocytosis shutdown

Since CLC degradation was an early event in apoptosis induction (Figure 2B and 3B), we suspected that inhibition of CLC degradation could affect endocytosis shutdown and apoptosis induction. The sequence of CLC contains the cyclin B-like sequence known as a destruction-box (D-box), a common motif for recognition by E3 ligases for

ubiquitinylation of proteins destined for proteasomal degradation [33]. This motif was mutated in CLC a-chain ($R^{205}SVL^{208}$ to ASVA, CLC D-box^{mut}) to inhibit recognition by ligases. In NB4 cells retrovirally transfected with CLC D-box^{mut}, CLC was prevented from degradation upon anthracycline treatment (Figure 6A). However, judged by immunoblotting, the endogenous CLC was not completely replaced by CLC D-box^{mut}, indicating that we are studying the effect of a mixed population of wildtype (wt) and D-box^{mut} CLC. Interestingly, by mutating only CLC a-chain, we also abrogated the degradation of CLC b-chain, supporting evidence that the b-chain is dependent upon the a-chain for stability [27].

To see whether the CLC D-box^{mut} clone displayed a different phenotype than the clone carrying wt CLC, we studied apoptosis induction and effects on endocytosis. The presence of CLC D-box^{mut} did not influence the rate of apoptosis induction when compared to the CLC wt clone (not shown). However, the CLC D-box^{mut} clone internalized transferrin to a greater extent after IDA treatment than the clone carrying CLC wt, indicating that the presence of CLC supports endocytosis after IDA-treatment (Figure 6B). After IDA treatment, the CLC D-box^{mut} clone also showed less accumulation of Fas-R (Figure 6C) and IFNγ-R (Figure 6D) compared to the CLC wt clone, suggesting that lack of CLC degradation after IDA facilitated persisting transferrin endocytosis as well as receptor accumulation at the cell surface.

Discussion

Clathrin-mediated endocytosis is essential for the cell both through receptor mediated uptake of nutrients and through regulation of receptor mediated signaling by receptor

internalization. In the present study we report that several proteins involved in clathrin-mediated endocytosis are targeted for caspase- or proteasomal-mediated degradation in anthracycline-induced AML cell death. We also show modulation of surface receptor expression and signaling in response to drug treatment. As drug-induced modulation of endocytosis was also seen in blasts from AML patients receiving induction therapy, we postulate that these effects could be of relevance *in vivo*.

A variety of proteins involved in different stages of clathrin-mediated endocytosis was targeted for cleavage or degradation in anthracycline-induced apoptosis. Formation of the clathrin triskelion was targeted through the degradation of CLC and cleavage of CHC and the adaptor proteins AP180, AP2-α and Eps15 [34,35]. Scission of clathrin coated vesicles was targeted by dynamin cleavage [36]. Also endosome fusion is targeted through cleavage of EEA1 [37]. CIN85, involved in regulation of receptor tyrosine kinase internalization through its interaction with the ubquitin ligase cbl, [38] was degraded in anthracycline-treated cells. Our data suggest that CLC is among the first proteins targeted, and based on morphological analysis and evaluation of procaspase-3 and selected targets we propose that CLC is degraded in a pre-apoptotic phase. This is supported by the observation that CLC is degraded in AML cells *in vivo*, where early apoptotic cells are eradicated from the circulation [26].

siRNA studies have reported somewhat contradictory results regarding the effects of knocking down these proteins on clathrin-mediated endocytosis. While a dominant negative Eps15 totally inhibited vesicular stomatitis virus entry by a clathrin-mediated pathway [39], siRNA knock-down of Eps15 did only to a small extent reduce endocytosis [27]. Neither did knock-down of CLC, EEA1 or CALM, the non-neuronal version of AP180,

severely inhibit TfR endocytosis [27], even though CALM to some extent inhibited EGFR internalization. However, siRNA knockdown of CLC inhibited entering of respiratory syncytical virus into the cells via clathrin-mediated endocytosis [40]. siRNA depletion of dynamin, CHC and AP2-α severely inhibited both constitutive TfR endocytosis and ligand-induced EGFR endocytosis [27,41,42]. On the other hand, other studies report neither CHC nor AP2-α to be essential for EGFR internalization, but only for transferrin uptake [43,44]. Targeting each single protein in itself might not be sufficient enough to completely inhibit clathrin-dependent endocytosis, but the simultaneous degradation and cleavage of several proteins would most likely ensure a more or less complete inhibition of clathrin-mediated endocytosis affecting both constitutive and ligand-induced endocytosis. The drug-induced decrease in transferrin uptake and accumulation of surface receptors supports this. Even thought we did not see any significant degradation of caveolin, the cleavage of dynamin [45] and Eps15 [46] will most likely also influence and impair modes of clathrin–independent endocytosis.

The mechanism by which anthracyclines induced CLC degradation is elusive. Based on our data we hypothesize that an ubiquitin and proteasome dependent mechanism is involved in CLC degradation. Anthracyclines activate the proteasome rapidly after administration [47], and proteasome activation has for etoposide induced apoptosis been shown to precede caspase activation [48]. The nature of the ligase responsible for CLC ubiquitinylation remains to be found, but e.g. Cbl, responsible for EGFR ubiquitinylation and found in the vicinity of clathrin coated vesicles [49], could be a candidate.

That CLC plays an essential role in clathrin-mediated endocytosis and is involved in pre-apoptotic modulation of endocytosis was supported by the effect on endocytosis of

disrupting the destruction-box in CLC. Mutating the D-box in CLC impaired its degradation and resulted in partially sustained transferrin internalization and decreased accumulation of surface receptors upon drug-treatment. The small extent to which CLC D-box^{mut} inhibits endocytosis shutdown probably reflects the time lag between CLC degradation and cleavage of other endocytic proteins.

The inhibitory effect on endocytosis correlated with accumulation of surface receptors such as TfR, Fas-R and IFNγ-R. As exocytosis is sustained pre-apoptotically in anthracycline-treated cells [50], the increased surface display is probably due both to decreased clathrin-mediated endocytosis and continued or induced recycling of receptors back to the surface. The accumulation of Fas-R and IFNγ-R were more prominent than the accumulation of TfR. In addition to being located to the cytoplasmic membrane, Fas-R is also present in cytoplasmic granules and is translocated from the cytosol to the cell surface upon death induction [51]. A similar intracellular pool of IFNγ-R also exists [52]. An induced translocation of intracellular receptor pools probably contributes to the prominent accumulation of these receptors.

While endocytosis has classically been viewed as a passive mean for terminating signaling, there is growing evidence that endocytic membrane trafficking plays a more sophisticated role in signaling from surface receptors [53]. The concept of "signaling endosomes" from which signal transduction continues after its initiation at the plasma membrane has proven important for EGFR signaling [54], nerve growth factor receptor signaling [55] and insulin receptor signaling [56]. Also internalization of the IFNγ-R might be a prerequisite for signaling and downstream activation of STATs [9]. Recruitment of the DISC and apoptosis induction after activation of Fas-R has also been reported to be

dependent upon internalization of the receptor [30]. We found that DNR co-treatment with IFNγ and Fas-monoclonal antibody enhanced apoptosis. This implies that death-signaling from the receptors is not dependent upon internalization and supports the findings of Austin et al. where Fas induced recruitment of FADD and caspase 8 and led to the activation of caspase 8 under conditions where endocytosis was blocked [31].

The finding that anti-apoptotic STAT5 [57] activation from IFNγ-R was abrogated after DNR-treatment and that this coincided with reduced transferrin endocytosis and surface accumulation of IFNγ-R, indicates that anti-apoptotic signaling from the receptor is dependent upon internalization. That anti-apoptotic signaling is dependent upon endocytosis has been reported as depletion of CHC induced apoptosis [58]. Reduced STAT5 activation could solely be due to decreased receptor endocytosis, but also to disruption/cleavage of proteins essential for an intermediate step in signal transduction, such as e.g. CIN85, shown to modulate both EGFR [59] and TNF-α signaling [60], or activation of phosphatases that can dephosphorylate STAT directly [61,62]. Reduced survival signaling mediated through STAT5 could be involved in the death enhancing effect of IFNγ on DNR-induced apoptosis.

Several surface receptors are preferentially expressed on cancer cells making them attractive candidates for targeted delivery of drugs. Both the TfR and the folate receptor type beta are selectively upregulated on AML cells and are therefore considered attractive candidates for targeted liposomal drug delivery [63-65]. Based on the findings in this work, we will call for caution in combining anthracyclines with drugs that depend upon internalization by clathrin dependent receptor endocytosis.

The present study shows that several components of the endocytotic machinery as well endocytosis itself is targeted and inhibited by anthracycline treatment. The drug-induced accumulation of surface receptors and decreased endocytosis is also seen in patients receiving anthracycline-based chemotherapy. Activation of surface receptors did have a modulating effect on anthracycline-induced apoptosis indicating that in the presence of ligands, their drug-induced accumulation could play a role in enhancement of anthracycline-induced apoptosis. Finally, this indicate CLC and the endocytotic machinery as a potential therapeutic target

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FIGURE LEGENDS

Figure 1. Anthracyclines induce degradation of clatrin light chain (CLC) and inhibit transferrin endocytosis in HL60 cells. (a) Cells were treated with vehicle (Ctr) or daunorubicin (DNR; 8 μM, 6 h), analyzed by 2DE and protein spots visualized by Sypro Ruby staining. MS-analysis verified a drug-induced degradation of CLC (accession number NM_001833; encircled). (**b**) Autoradiograms of proteins translated *in vitro* in the presence of [^{35}S]-methionine with RNA isolated from vehicle or DNR (8 μM, 4.5 h) treated cells as template. (**c**) Cells were pre-labeled with [^{35}S]-methionine and chased for 6.5 h with unlabeled methionine with vehicle or DNR (8 μM) present during the last 6 h of incubation. Note the disappearance of pre-labeled CLC. (**d**) Cells were treated with DNR (8 μM, 2 or 5 h) with Alexis 488-conjugated transferrin present the last 10 min. The cells were processed for scanning confocal microscopy and transferrin uptake was studied. The apoptotic state of the cell was determined by nuclear chromatin DNR fluorescence. The experiment shown is representative of five experiments. Magnification: 900x. (**e**) FACS-analysis of transferrin endocytosis in IDA (0.72 μM, 5 h) treated cells. Only cells with a forward and side scatter identical to vehicle treated cells were analyzed for transferring Alexis 647 endocytosis (**f**) The two cell populations in (e) showing different transferrin endocytosis were analyzed for surface expression of the transferrin receptor (TfR). The experiment is representative of 3.

Figure 2. Anthracycline-induced CLC degradation and modified endocytosis happen pre-apoptotically in AML blasts *in vivo*. (**a**) 2DE analysis of protein extracts

from i*n vitro* treated AML blasts (8 μM DNR, 6 h) isolated from two patients (#1, #2). (**b**) AML blasts from three patients (#3-#5) were collected before and 4 h, 16 h or 24 h respectively after onset of induction treatment with IDA and AraC, and cell extracts were analyzed by 2DE. The encircled spot represents CLC. Note the decrease/disappearance of CLC after induction treatment. All gels were silver stained. (**c-e**) AML blasts from two patients (#6: closed bars, #7: open bars) were collected at times indicated after start of induction treatment with IDA and AraC, and blasts were assayed for internalized transferrin (c), surface expression of Fas-R/CD95 (d) and labeled with AnnexinV to assay cell viability (d). Patient numbers refer to Supplementary Table 1.

Figure 3. Anthracyclines induce cleavage/degradation of proteins involved in clathrin-dependent endocytosis. **(a)** HL60 cells were treated with DNR (8 μM, 5 h, 50 % apoptosis) and cell extracts were immunoblotted and probed for proteins involved in clathrin-mediated endocytosis. **(b,c)** HL60 cells were treated for 1 to 6 h with DNR (1.6 μM), and cell extracts were immunoblotted and probed against proteins involved in clathrin-dependent and -independent endocytosis (b) and scored for apoptosis (c).

Figure 4. Clathrin-dependent endocytosis is targeted through caspase- and proteasomal-mediated degradation of structural and regulatory proteins. (a) HL60 cells were treated with IDA (0.72 μM, 5 h) or DNR (8 μM, 4 h) in the presence or absence of zVAD-fmk (100 μM given 15 min before addition of the anthracycline). Cell extracts treated with IDA were immunoblotted and probed against CLC, Eps15, AP2-α and CIN85, and cell extracts treated with DNR were probed against EEA1, dynamin and

procaspase-3. **(b)** HL60 cells were treated for 15 min with MG132 before addition of IDA (0.72 μM, 5 h) and cell extracts immunoblotted and probed for CLC (1 μM MG132) and AP2-α (1 μM MG132). **(c)** Cells were treated with vehicle or DNR (8 μM, 5 h) in the presence or absence of MG132 (30 μM) or zVAD (100 μM) and analyzed by FACS for transferrin endocytosis.

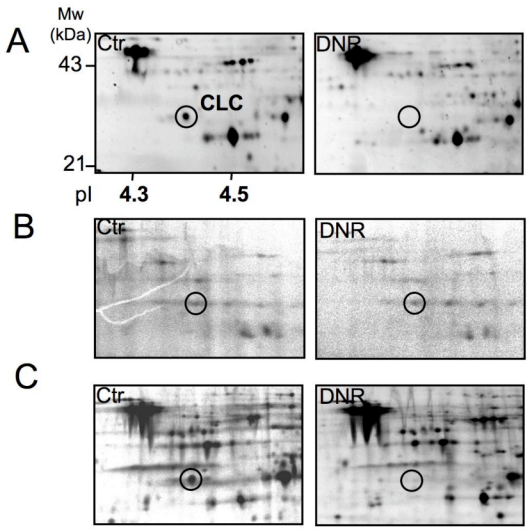
Figure 5. Surface receptor expression and signaling are modified during anthracycline treatment. FACS-analysis of Fas-R/CD95 (**a**) and IFNγ-R/CD119 in NB4 cells treated with vehicle, IDA (720 nM, 5 h) or IDA + cycloheximide (CHX; 3.6 μM). **(c)** HL60 cells were treated with vehicle or IDA (720 nM, 5 h) and stimulated with IFNγ (20 ng/ml) the last 15 min. Transferrin Alexis 488 were present the last 10 min, and P-STAT5 were analyzed by FASC. For IDA treated cells, the two populations differing in transferrin endocytosis (reduced endocytosis, shown, and sustained endocytosis, not shown and identical to Ctr) were separately analyzed for P-STAT5. The experiment shown is representative of 3. **(d)** Cells were treated with vehicle or DNR (1.6 μM, 4 h) in the presence or absence of monoclonal antibody towards Fas-R (1 μg/ml) or IFNγ (20 ng/ml), and apoptosis was scored. Data represents ±SEM of 3 experiments. For further details, se Materials and Methods.

Figure 6. Inhibition of CLC degradation maintains clathrin-dependent endocytosis after anthracycline treatment. (a) NB4 cells stably transfected with CLC wt and CLC carrying the mutations R205A and L208A (CLC D-box^{mut}) were treated with vehicle, DNR (5 μM, 5 h) or IDA (0.5 μM, 5 h) and cell extracts were analyzed for CLC by

immunoblotting. **(b)** CLC wt and D-box^{mut} were treated with IDA (0.72 μM, 5 h) and transferrin Alexis 647 were present during the last 10 min. Cells were analyzed by FACS for transferrin endocytosis. Data shown are representative of 4 experiments. **(c,d)** CLC wt and D-box^{mut} were treated with IDA (0.72 μM, 5 h) and analyzed by FACS for surface expression of Fas-R (c) and IFNγ-R (d).

Supplementary Table 1. Clinical and biological characteristics of patients. Abbreviations: female (F), male (M), acute myelogenous leukemia (AML), Flt3 length mutation (Flt3 LM), nucleophosmin (NPM-1) nd: not determined.

Figure 1


A
Mw
(kDa)
43
21
pI
4.3
4.5
Ctr
DNR
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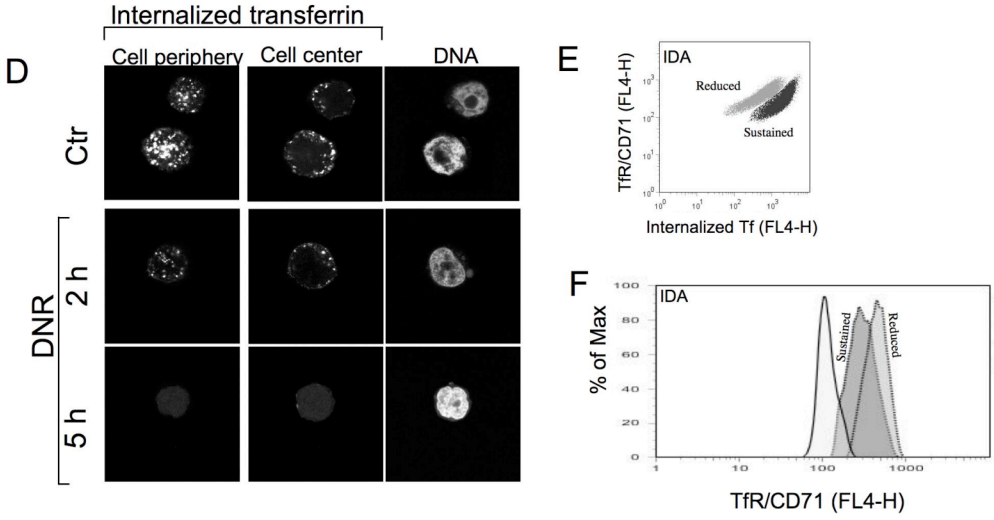

D
Internalized transferrin
Cell periphery
Cell center
DNA
Ctr
DNR
2 h
5 h
E
IDA
Reduced
Sustained
TfR/CD71 (FL4-H)
Internalized Tf (FL4-H)
F
IDA
% of Max
Sustained
Reduced
TfR/CD71 (FL4-H)

Figure 2

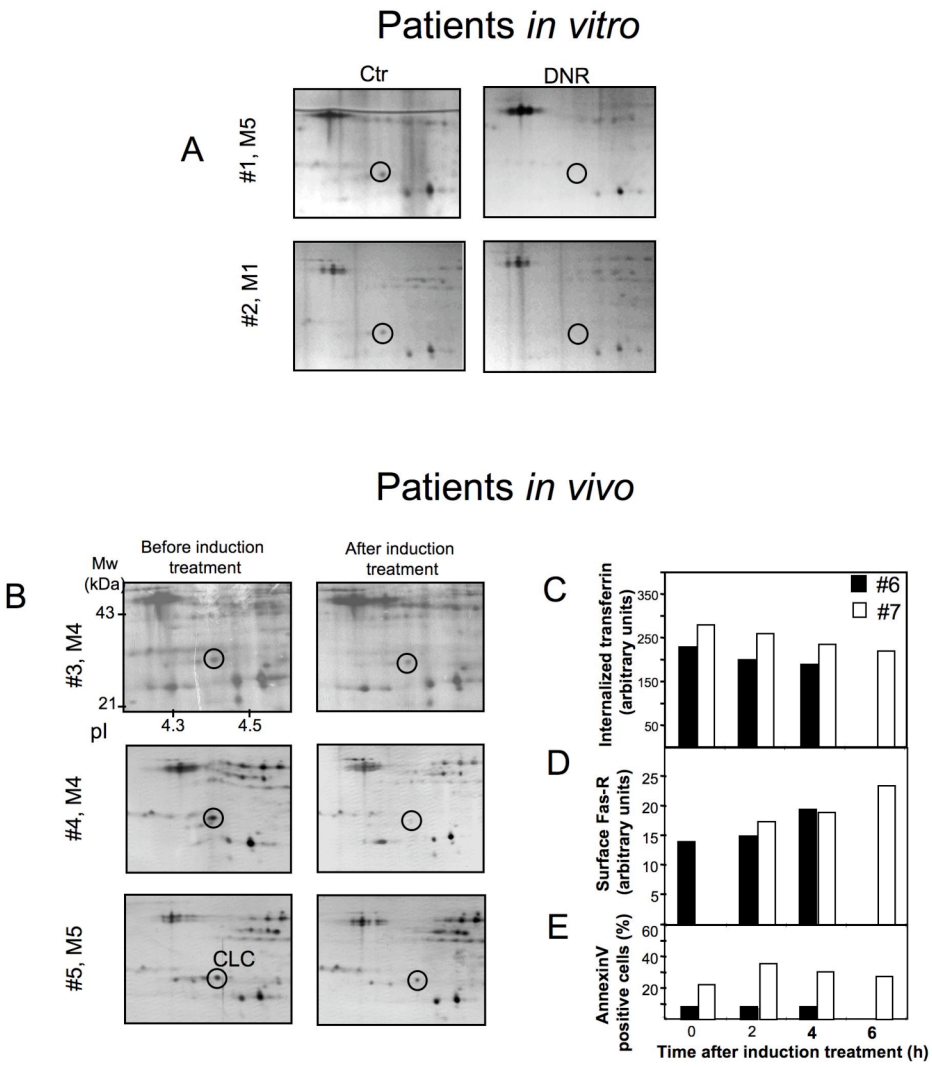

Patients *in vitro*
A
Ctr
DNR
#1, M5
#2, M1
Patients *in vivo*
B
Mw (kDa)
Before induction treatment
After induction treatment
43
21
pI
4.3
4.5
#3, M4
#4, M4
#5, M5
CLC
C
#6
#7
Internalized transferrin (arbitrary units)
350
250
150
50
D
Surface Fas-R (arbitrary units)
25
20
15
10
5
E
AnnexinV positive cells (%)
60
40
20
0
2
4
6
Time after induction treatment (h)

Figure 3

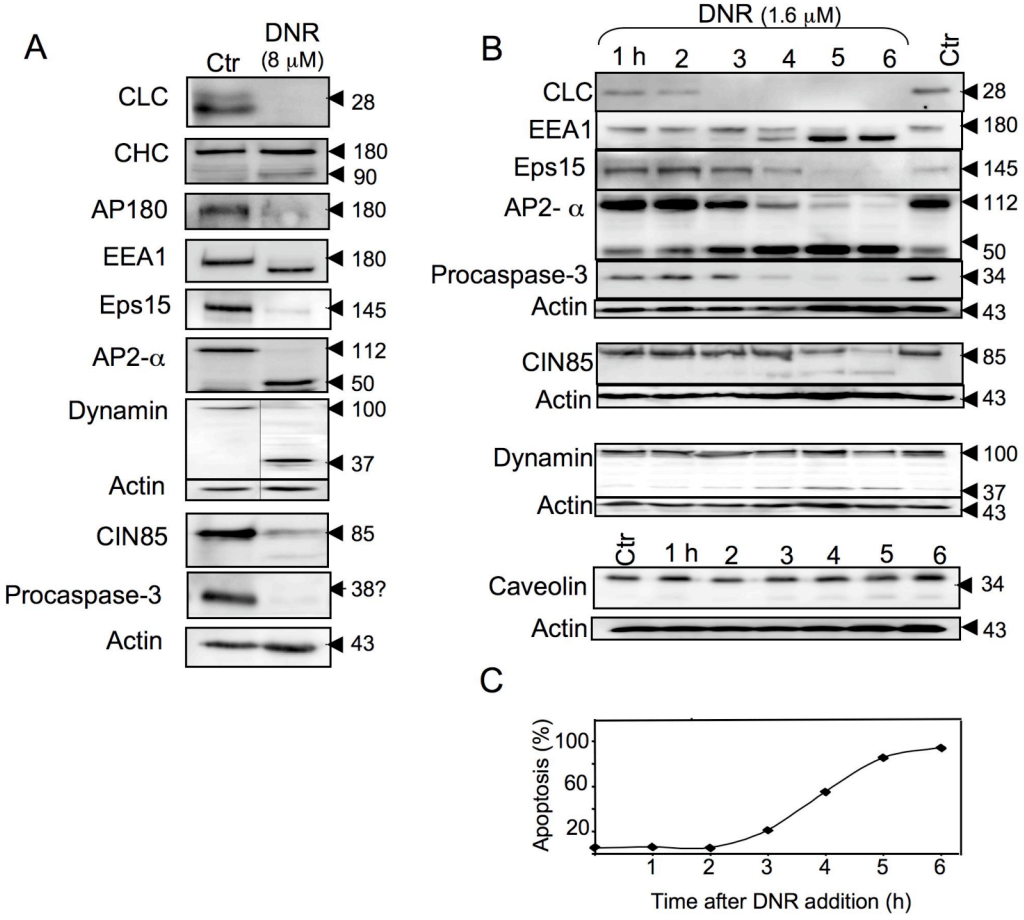

A
Ctr
DNR (8 μM)
CLC
28
CHC
180
90
AP180
180
EEA1
180
Eps15
145
AP2-α
112
50
Dynamin
100
37
Actin
43
CIN85
85
Procaspase-3
38?
Actin
43
B
DNR (1.6 μM)
1 h
2
3
4
5
6
Ctr
CLC
28
EEA1
180
Eps15
145
AP2- α
112
50
Procaspase-3
34
Actin
43
CIN85
85
Actin
43
Dynamin
100
Actin
37
43
Ctr
1 h
2
3
4
5
6
Caveolin
34
Actin
43
C
Apoptosis (%)
100
60
20
1
2
3
4
5
6
Time after DNR addition (h)

Figure 4

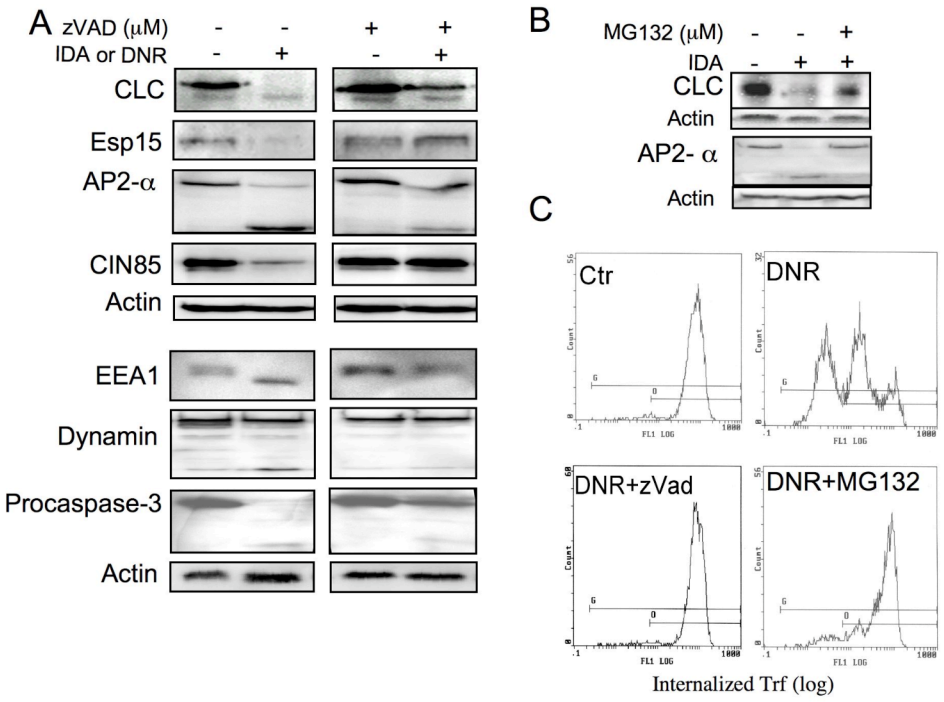

A
zVAD (μM) - - + +
IDA or DNR - + - +
CLC
Esp15
AP2-α
CIN85
Actin
EEA1
Dynamin
Procaspase-3
Actin
B
MG132 (μM) - - +
IDA - + +
CLC
Actin
AP2- α
Actin
C
Ctr
DNR
DNR+zVad
DNR+MG132
Internalized Trf (log)

Figure 5

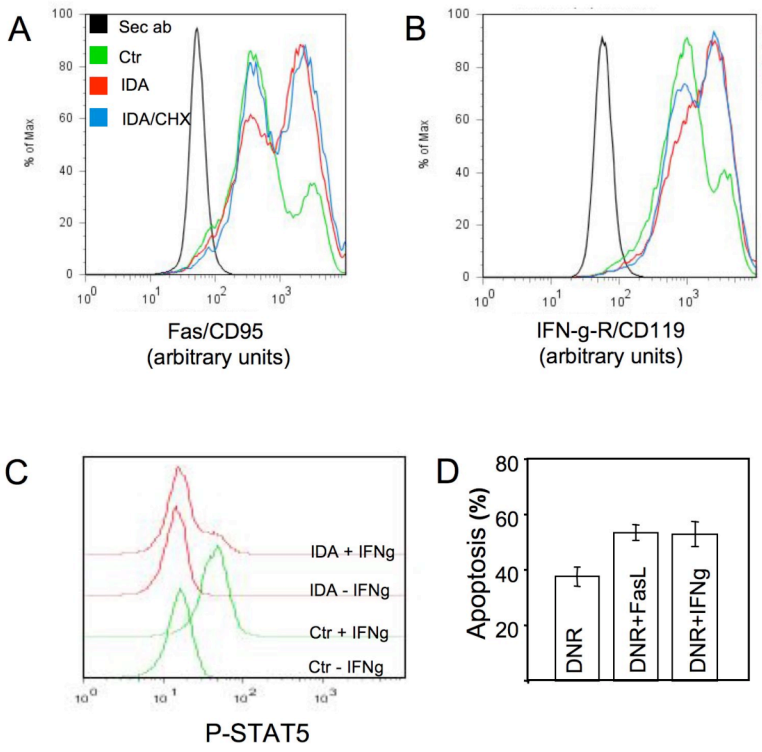
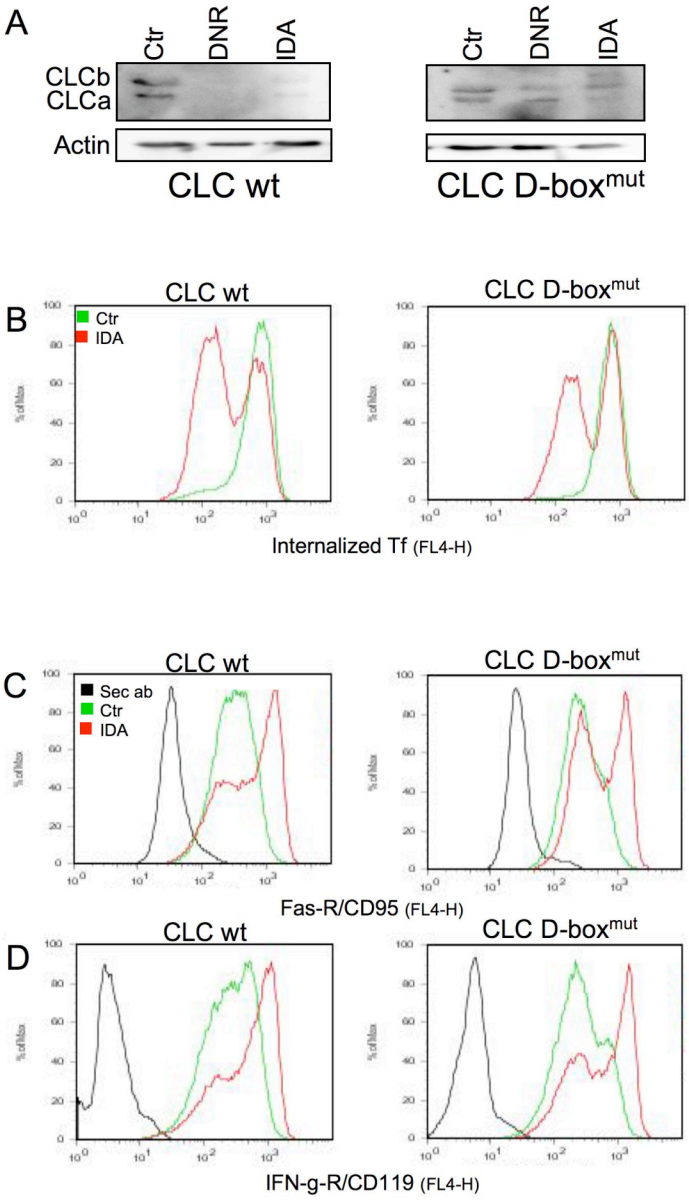

A
Sec ab
Ctr
IDA
IDA/CHX
% of Max
Fas/CD95
(arbitrary units)
B
% of Max
IFN-g-R/CD119
(arbitrary units)
C
IDA + IFNg
IDA - IFNg
Ctr + IFNg
Ctr - IFNg
P-STAT5
D
Apoptosis (%)
DNR
DNR+FasL
DNR+IFNg

Figure 6


A
Ctr
DNR
IDA
CLCb
CLCa
Actin
CLC wt
CLC D-box^mut
B
CLC wt
CLC D-box^mut
Ctr
IDA
Internalized Tf (FL4-H)
C
CLC wt
CLC D-box^mut
Sec ab
Ctr
IDA
Fas-R/CD95 (FL4-H)
D
CLC wt
CLC D-box^mut
IFN-g-R/CD119 (FL4-H)

Supplementary Table 1. Clinical and biological characteristics of patients

Patient	Age	Sex	Classification	Membrane molecules						**Flt3 LM**	**Flt Asp835**	**NPM-1**
				CD13	CD14	CD15	CD33	CD34	Karyotype			
1	82	M	AML-M5	(-)	(-)	(+)	(+)	(-)	45X	wt	wt	wt
2	45	F	AML-M1	nd	nd	nd	nd	nd	Normal	nd	nd	nd
3	29	M	AML-M4	(+)	(-)	(-)	(+)	(+)	Normal	0,06	0,17	+
4	63	F	AML-M4	(+)	(-)	(-)	(+)	(+)	Normal	0,04	wt	nd
5	82	M	AML-M5	(+)	(+)	(+)	(+)	(-)	Normal	wt	wt	+
6	55	M	Atypical	(+)	(-)	(+)	(+)	(-)	Multiple	wt	wt	nd
7	61	M	AML-M4/M5	(-)	(-)	(+)	(+)	(-)	Normal	0,03	nd	+