Optimized anti-tumor effects
of anthracyclines plus vinca alkaloids
using a novel, mechanism-based application schedule

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running title: optimized schedule for doxo plus VCR
Abstract

Application of anthracyclines and vinca alkaloids on the same day represents a hallmark of polychemotherapy protocols for hematopoietic malignancies. Here we show for the first time that both drugs might act most efficiently, if they are applied on different days. Proof-of-concept studies in 18 cell lines revealed that anthracyclines inhibited cell death by vinca alkaloids in 83% of cell lines. Importantly, in a preclinical mouse model, doxorubicine reduced the anti-tumor effect of vincristine. Both drugs acted in a sequence-dependent manner and the strongest anti-tumor effect was obtained, if both drugs were applied on different days. Most notably for clinical relevance, in 34% of 35 fresh primary childhood leukemia cells tested in vitro, doxorubicine reduced the anti-tumor effect of vincristine. As underlying mechanism, doxorubicin activated p53; p53 induced cell cycle arrest; cell cycle arrest disabled inactivation of anti-apoptotic Bcl-2 family members by vincristine; therefore, vincristine was unable to activate downstream apoptosis signaling. As molecular proof, antagonism was rescued by knockdown of p53, while knockdown of cyclinA inhibited vincristine-induced apoptosis. Our data suggest evaluating anthracyclines and vinca alkaloids on different days in future trials. Selecting drug combinations based on mechanistic understanding represents a novel conceptional strategy for potent polychemotherapy protocols.


Introduction

Chemotherapeutic drugs such as anthracyclines and vinca alkaloids are used during anti-tumor therapy with the aim of clearing tumor cells upon induction of cell death. As mono-therapy is poorly effective, chemotherapeutic drugs are combined in polychemotherapy protocols in order to increase anti-tumor efficiency (1-3).

Combinations of different chemotherapeutic drugs have been optimized heuristically in clinical multicenter trials. Due to substantial efforts required to realize clinical trials, only a limited number of drug combinations and application parameters could be optimized clinically. For example, anthracyclines and vinca alkaloids are widely co-applied on the same day in many different anti-cancer protocols to treat hematopoietic malignancies and some forms of solid tumors. Nevertheless, the application schedule and sequence of these two classes of chemotherapeutic drugs was never optimized in clinical trials (4,5). The rational for using both drugs in combination was based on very limited (< 10) animal experiments back in the 1970th which were performed exclusively on rat and mouse tumor cells, as far as we know (6,7).

For certain chemotherapeutic drug combinations, anti-tumor efficiency highly depends on the application schedule, as shown in few clinical trials and numerous preclinical studies (8-13). Among others, sequence dependency was proven for the combination of asparaginase and methotrexate for anti-leukemia therapy (8,10,12-16).

On a molecular level, the effects and signaling pathways induced by many chemotherapeutic drugs have not been analyzed in depth and the consequences of their combinatorial application remain unclear (17). As far as we know, no mechanistic data exist so far to explain the sequence dependency of any clinically proven drug combination on a molecular level.

Here, we aimed at optimizing the anti-tumor effect of the drug combination of anthracyclines and vinca alkaloids based on an understanding of their signaling interactions. We detected a strongly sequence dependent anti-tumor effect of both drugs and characterized the intracellular signaling mechanisms responsible for sequence dependency in leukemia cells. We introduced an optimized, mechanism-based application schedule which might improve the effectiveness of both drugs in clinical trials for hematopoietic malignancies.
Methods

Materials
For Western blot, the following antibodies were used: anti-Bcl-xL, anti-Casp-2, anti-cleaved Casp-3, anti-cleaved Casp-6, anti-cleaved Casp-7, anti-cleaved PARP and anti-phospho Histone H3 (Ser10) from Cell Signaling Technology (Danvers, MA); anti-Bcl-2, anti-cleaved Casp-1 and anti-p53 from Santa Cruz (Santa Cruz, CA); anti-cyclinA and anti-GAPDH from Thermo Fisher (Waltham, MA); anti-Casp-9 from Transduction Laboratories (San Diego, CA); anti Casp-10 from MBL (Watertown, MA); anti-caspase-8 from Alexis Corp (Lausen, Switzerland). For flow cytometric analysis Annexin V was obtained from BD Biosciences (Franklin Lakes, NJ) and anti-p-Histon H3 Ser 10 from Cell Signaling Technology. Vincristine and all biochemical inhibitors were obtained from Calbiochem (Darmstadt, Germany) with the exception of 2,3-DCPE (Biomol International LP, Plymouth Meeting, PA). All other reagents were obtained from Sigma (St. Louis, MO).

Cell lines, transfection experiments and primary samples
All leukemia cell lines were obtained from DSMZ (Braunschweig, Germany) and were maintained as previously described (18,19). For all cell line experiments, cells were seeded at 0.2x10^6/ml and incubated with chemotherapeutic drugs at peak plasma concentration for 48 hours unless otherwise stated. CFUs were performed with a starting cell density of 0.02x10^6/ml using methylcellulose base media supplemented with 2mM L-glutamine, 10mM HEPES buffer solution and 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA). Transfection experiments where performed using the Cell Line Nucleofector kit V (Lonza, Walkersville, MD) according to the manufacturers’ instructions and shRNA against p53 and mock plasmids as previously described (19). Alternatively, lentiviral transduction was performed targeting the identical p53 sequence (paper submitted) or cyclin A with the following oligonucleotides annealed: sense sequence 5’-GATCCGAAATGTACCCCTCAGAAATTGAATTCGTTTCTGGAGGATCATTTCTTTTTTG-3’, antisense sequence 5’-AATTCAAAAAGAAATGTACCCTCCAGAAACGAATTCAATTTCTGGAGGATCATTTCTTTTG-3’.
Primary leukemia blasts were obtained from 35 children treated for acute leukemia at the Ludwig Maximilian University Children’s Hospital and the children’s hospital of TU
Munich during 2005 and 2008. Samples were obtained, isolated and stimulated simultaneously with doxorubicin and vincristine as previously described (19,20).

**Animal trial**
The animal trial was approved by the Bavarian federal government and animal care was in accordance with institution guidelines. Female NSG mice aged 8-12 weeks were obtained from Charles River. Mice were subcutaneously injected with 0.1 ml containing $2.8 \times 10^6$ CEM cells into both flanks. After 10 days, all animal had developed tumors with diameters ranging between 2 and 6 mm. The animals were distributed into groups (control, n=8 animals; doxo, n=8; VCR, n=16; doxo+VCR, n=19) and treated with i.v. injections of doxorubicin solution (0.3 mg/kg body weight; MEDAC, Wedel, Germany) and/or the i.v. administration of vincristine solution (0.9 mg/kg body weight; TEVA, North Wales, PA) at day 0 and 8. Tumor size (two dimensions) was determined at the beginning of treatment and was followed for 15 days. The relative change in tumor size was calculated for each animal.

**Cell imaging, flow cytometric analysis, apoptosis assays, and Western blot analysis**
For biochemical inhibition, cell lines were pretreated for 8 hours or irradiated 24 hours before further stimulation for another 48 hours. The release of cytochrome c was detected as recently described (18), loss of mitochondrial membrane potential using DiOC$_6$ staining. Cell cycle analysis was performed using PI-staining. To discriminate between G2 and M arrest, double staining for p-Histon H3 and propidium iodid was performed. In brief, cells were fixed by 70% ethanol, resuspended in PBS with 0,25% Triton X, followed by incubation with specific antibody in PBS with 1% BSA and addition of RNAse A (100µg/ml) and propidium iodid 20µg/ml after three washing steps. Apoptosis was measured by forward side scatter analysis and precision of this technique confirmed by Annexin V and propidium iodid double staining according to the manufacturers instructions using FACscan or LSR II flow cytometry and Cell Quest Pro (BD Biosciences) and FlowJo (FlowJo, Ashland, OR) software. Western blot analysis of total cellular protein or of cytosolic and nuclear fractions was performed as previously described and 10µg of protein were loaded (19).
**Statistical analysis**

Specific apoptosis was calculated as \[(\text{apoptosis of stimulated cells at end} - \text{apoptosis of unstimulated cells at end}) \div (100 - \text{apoptosis of unstimulated cells at end}) \times 100\], specific survival as \[100 - \text{specific apoptosis induction}\]. In Figure 2C, doxorubicin resistance was defined as specific apoptosis of less than 10 %. In Figure 2C, Fractional product method (FP; 21) was employed to discriminate between synergistic and antagonistic apoptosis induction after combined application of doxo and VCR. FP values \(\leq -0.1\) were defined as relevant antagonism, FP values \(\geq 0.1\) as relevant synergism. For primary samples, the expected apoptosis induction of independent application of doxo and VCR was calculated as \[(1 - (\text{survival after simulation with doxo} \times \text{survival after stimulation with VCR})) \times 100\]. Alternatively, median effect blots were used performed by CompuSyn software. For cell line experiments, data are presented as the mean values of at least three independent experiments \(\pm\) SEM unless otherwise stated. To test for significant differences, the paired t-test was applied; for multivariate analysis, one way RM ANOVA was used. Significance was set at \(p < 0.05\). For animal trials, Mann Whitney rank sum test or student’s t-test was applied for \(p < 0.01\).
Results

Anthracyclines and vinca alkaloids are applied on the same day in several polychemotherapy protocols for hematopoietic malignancies. Here, we aimed at optimizing their anti-tumor effect based on the understanding of the responsible signaling interaction.

Inhibition of vinca alkaloid-induced apoptosis by anthracyclines

The T-cell leukemia cell lines CEM (data presented in printed Figures and suppl. Figures 1-3) and JURKAT (data presented in suppl. Figure 4) are highly sensitive towards vincristine (VCR)-induced apoptosis and partially sensitive towards doxorubicin (doxo)-induced apoptosis. To our surprise and as a completely new finding, doxo inhibited VCR-induced apoptosis in both cell lines, when both drugs were given simultaneously (Figure 1A). The net effect of the combinatorial use of both drugs resulted in antagonism as measured by various techniques including morphology, forward side scatter analysis in FACscan, Annexin V staining, uptake of propidium iodide and DNA fragmentation (suppl. Figure 1). Both median effect plots and the fractional product method confirmed antagonistic interaction between doxo and VCR (Figure 1B and data not shown). More detailed studies revealed that doxo inhibited VCR-induced apoptosis over a long period of time, in a dose-dependent manner and enabled the survival of colony-forming tumor cells otherwise erased by VCR (Table 1 and suppl. Table 1). The anti-apoptotic effect of doxo was not due to direct drug-drug interaction as it persisted after medium exchange. The anti-apoptotic effect was not restricted to doxo, but also observed with other anthracyclines including daunorubicin, epirubicin, and idarubicin. Reciprocally, doxo attenuated induction of cell death not only by VCR, but also by vinblastine and vinorelbine, suggesting a general inhibitory and anti-apoptotic effect of anthracyclines towards vinca alkaloid-induced cell death (data not shown for all).

Cell lines of different hematopoietic tumors were tested, including 8 B- and T-ALL cell lines, 7 AML cell lines and 3 lymphoma cell lines. All lines expressed functionally active p53 (22). Overall in 15 out of 18 (83%) cell lines, a negative interaction between doxo and VCR was detected, when both drugs were applied together, as their mutual apoptosis induction was lower than expected from the activity from the single drugs (Figure 1A, C-E and suppl. Figure 4A). When
hematopoietic subtypes were analyzed, inhibition of VCR-induced apoptosis was present in 63% of ALL and 100% of AML and lymphoma cell lines.

To test the described phenotype within the complex in vivo situation, NSG mice were xenografted subcutaneously with human CEM T-ALL leukemia cells. CEM cells bearing mice were treated with either doxo or VCR alone or both drugs simultaneously. Similar to the in vitro data, doxo significantly inhibited the anti-tumor effect of VCR in vivo, when doxo was applied together with VCR (Figure 1F).

In summary, doxo frequently and severely inhibited VCR-induced apoptosis in hematopoietic tumor cells in vitro and in vivo when both drugs were applied simultaneously.

**Antagonistic effect of doxorubicin and vincristine on primary leukemic tumor cells**

To approximate the clinical situation and move beyond established cell lines, 35 fresh primary tumor samples were investigated. Cells were obtained from children with acute leukemia and isolated from diagnostic bone marrow aspirations before the onset of anti-cancer treatment. Fresh primary leukemia cells were stimulated in vitro with each drug alone or both drugs simultaneously applying a range of clinically relevant drug concentrations. Doxo inhibited VCR-induced apoptosis in several primary leukemia tumor cells (Figure 2A) and over a broad range of concentrations (Figure 2B).

Next we aimed to estimate the effect of the different application schedules of both drugs on a number of primary leukemia cells. Unfortunately, these cells allow only one single short-time experiment in vitro. To estimate, how efficient the two drugs might have been, if they had been given one after the other, independently from each other, independent apoptosis induction was calculated out of the data obtained by single agent stimulation using the equation by Webb (Figure 2C, white and grey bars; details of calculations in Methods). This equation allows the most precise calculation on limited numbers of experiments (21) (Figure 2C, white and grey bars; details of calculations in Methods). For comparison, apoptosis induction by the drug combination is depicted as measured as black dots in Figure 2C. In 11% of samples, apoptosis induction was increased by simultaneous application of doxo and VCR compared to independent application yielding synergistic apoptosis. In contrast, simultaneous application of doxo and VCR was less effective than independent
application yielding antagonistic apoptosis: When both drugs were given at peak plasma concentration, doxo inhibited VCR-induced apoptosis in 34% (12/35) of samples which increased to 54% (19/35) of samples, when lower concentrations of doxo were included (data not shown). Within the small cohort, no correlation of inhibition of VCR-induced apoptosis by doxo with genetic alterations could be detected (data not shown).

In summary, doxo frequently and markedly inhibited VCR-induced apoptosis in primary tumor cells from children. Importantly, doxo exerted its anti-apoptotic effect on leukemia cells obtained from those children who received simultaneous application of doxo and VCR within induction therapy of the ongoing polychemotherapy trial.

Sequence dependency of the antagonism in vitro and in vivo

When two or more chemotherapeutic drugs are combined, sequence dependent effects were revealed for a number of drug combinations both in vitro as well as in clinical studies (8,10,12-16). Therefore, we studied, whether the antagonism between doxorubicin and vincristine might be sequence dependent. Indeed, when VCR was given first, 1 day before doxo, VCR induced significant apoptosis. In contrast, when doxo was given first or together with VCR, doxo markedly inhibited apoptosis induction by VCR (Figure 1B, 3A and B). At least in part, doxo reduced VCR-induced apoptosis even when given after VCR (suppl. Figure 4E). Thus, the efficiency of the combinatory treatment of doxo and VCR depends on the sequence of application and whether doxo encounters active VCR.

Taken together, doxo inhibited apoptosis induction by VCR depending on the application schedule. These data add the clinical routine combination of anthracyclines and vinca-alkaloids to the drug combinations with sequence dependent anti-tumor effects.

Signaling mechanism of VCR and inhibition by doxo

To characterize underlying signaling mechanisms responsible for the negative drug interaction, doxo and VCR were studied in parallel on CEM and JURKAT leukemia cell lines.

First, we aimed to characterize the effects of doxo on VCR-induced apoptosis signaling events. The apoptosis signaling cascade activated by VCR is not
completely characterized, but it involves incomplete spindle formation, cell cycle
arrest, activation of p53 and NF-kappaB, phosphorylation of anti-apoptotic Bcl-2
members, and initiation of the downstream intrinsic apoptosis signaling cascade,
among others (23). In a candidate approach, we analyzed the expression levels and
functions of putative players.

VCR activates the intrinsic apoptosis signaling cascade. The Bcl-2 and IAP-
member families contain important antagonists of this signal transduction. Their
expression levels remained unchanged by treatment with doxo, VCR or the
combination of both (suppl. Figure 2A). During VCR-induced apoptosis, anti-apoptotic
Bcl-2 and Bcl-xL become inactivated by phosphorylation (23). Apoptosis induction by
VCR alone highly depended on phosphorylation of anti-apoptotic Bcl-2 members, as
overexpression of phosphorylation deficient mutants of Bcl-2 and Bcl-xL markedly
reduced apoptosis induction by VCR (24, 25; suppl. Figure 2B). For the combined
application of doxo and VCR, the phosphorylation of Bcl-2 and Bcl-xL by VCR was
markedly inhibited by doxo (Figure 4A). Thus, failure to phosphorylate and inactivate
anti-apoptotic Bcl-2 and Bcl-xL was identified as the most proximal signaling step
within the VCR-induced apoptotic signaling cascade, which was inhibited by doxo.
After lack of phosphorylation and inactivation of Bcl-2 and Bcl-xL, all downstream cell
death signaling steps which were rapidly activated upon VCR-induced apoptosis
were inhibited by doxo, including loss of mitochondrial membrane potential, release
of cytochrome c, cleavage of caspases, and cleavage of PARP (Figure 4B).

To study the role of Bcl-2 members in apoptosis inhibition in more detail, 2,3-
dichlorophenoxypropylaminoethanol (DCPE) was added which is known to
downregulate Bcl-2 family members by yet unknown mechanisms (26). DCPE
reduced the expression levels of anti-apoptotic Bcl-2 and Bcl-xL as shown by
Western Blot (Figure 4C). Thereby, DCPE enabled to override the inhibitory effect of
doxo on VCR-mediated apoptosis (Figure 4C). Treatment with two phosphatase-
inhibitors, calphostin c and okadaic acid, caused phosphorylation of Bcl-2 and Bcl-xL
independently from VCR by blocking de-phosphorylation (Western Blots in Figure 4C
and data not shown). Calphostin c and okadaic acid restored VCR-induced apoptosis
in the presence of doxo, supporting an important role for the stabilization of anti-
apoptotic Bcl-2 members in doxo-induced inhibition of apoptosis (Figure 4C and data
not shown).
Taken together, these results show that doxo stabilized anti-apoptotic Bcl-2 family members by preventing their phosphorylation which was reversed by treatment with DCPE, calphostin c, and okadaic acid. Doxo inhibited VCR-induced Bcl-2 member phosphorylation and thereby disabled the downstream intrinsic apoptosis signaling cascade of VCR (Figure 6E).

**Signaling mechanism of doxo and impact on VCR-induced apoptosis**

Next, we searched for signaling molecules that mediate the anti-apoptotic function of doxo. In tumor cells, anthracyclines are known to intercalate into DNA, induce formation of free radicals and inhibit topoisomerase II among other actions (27). Anthracyclines induce DNA damage, activate ataxia telangiectasia mutated kinase (ATM), induce p53 and lead to cell cycle arrest or apoptosis (27,28).

The transcription factor p53 is activated by DNA-damage and signals pleiotrophic effects, such as DNA repair, cell cycle arrest, and cell death (29). In our two cell lines investigated, p53 is present and doxo induced the accumulation of nuclear p53 both in the presence and absence of VCR (Figure 5A; data not shown). The downregulation of p53 protein levels restored VCR-induced apoptosis in the presence of doxo indicating an important role for p53 in this process (Figure 5B).

Activated p53 induces cell cycle arrest which might participate in apoptosis inhibition. Both VCR and doxo caused a marked accumulation of cells in G2/M (Figure 6A and suppl. Figure 3A). Further cell cycle discrimination revealed that doxo arrested the cell cycle in G2, while VCR arrested the cell cycle in M (Figure 6B). No signs of cellular senescence were detected (data not shown). When drugs were combined, cell cycle arrest was enhanced and more than 80 % of the cells were arrested in the G2 for the entire observation period available in cell culture (Figure 6A and data not shown). Cell cycle arrest in G2 by doxo was reduced in p53 knock-down cells (suppl. Figure 3B).

To prevent cell cycle arrest induced by doxo and VCR, cells were pretreated with caffeine or KU-55933, an inhibitor of ATM. Both compounds alleviated the block in the presence of both doxo and VCR (suppl. Figure 3C; data not shown) and sensitized the cells for VCR-induced apoptosis (Figure 6C and data not shown).

To arrest the cell cycle on a molecular level, cyclinA was knocked down by RNA interference which induced a major fraction of cells in G2 (Figure 6D). Knockdown of cyclinA and concomitant cell cycle arrest significantly inhibited VCR-
induced apoptosis (Figure 6D) proving that cell cycle arrest disabled VCR-induced apoptosis.

Taken together, these results show that p53 and cell cycle arrest mediate the anti-apoptotic function of doxo. Doxo inhibited VCR-induced apoptotic signaling upstream of Bcl-2 / Bcl-xL. Lack of p53, addition of two cell cycle stimulators, caffeine or KU55933, or addition of agents antagonizing Bcl-2 / Bcl-xL such as calphostin c, okadaic acid and DCPE alleviated the anti-apoptotic function of doxo towards VCR-induced apoptosis, while knockdown of cyclinA inhibited VCR-induced apoptosis similarly to doxo as illustrated in a schematic chart (Figure 6E).

**Cell cycle arrest-based antagonistic interaction between irradiation and vincristine**

So far, our mechanistic studies revealed that cell cycle arrest mediated doxo-induced inhibition of VCR-induced apoptosis. Based on these new mechanistic insights, we hypothesized that further cell-cycle arresting drugs and stimuli would inhibit VCR-induced apoptosis similarly to doxo.

Indeed, irradiation induced cell cycle arrest in G2 (30,31) and significantly inhibited VCR-induced cell death (Figure 7). Similarly to the phenotype observed in G2 arrested cells, cell cycle arrest in G0 or G1 by dexamethasone, serum starvation or L-Mimosine hampered apoptosis induction by VCR (data not shown). These data show that VCR is generally unable to induce cell death in cell cycle-arrested tumor cells and that VCR requires active cell cycling for effective induction of apoptosis. Thus, our new understanding of the mechanisms of VCR-induced apoptosis enabled the identification of numerous antagonizing stimuli.

Taken together, we have identified cell cycle arrest as a new general mechanism responsible for sequence-dependent effects of drug combinations with VCR: If VCR is given first and activates the pro-apoptotic signaling cascade before induction of cell cycle arrest by any second stimulus, VCR potently induces apoptosis. If a cell cycle arresting, cytostatic stimulus is given shortly before VCR, the pro-apoptotic efficacy of VCR is markedly reduced.
Discussion

In several different polychemotherapy protocols for hematopoietic tumors, anthracyclines and vinca-alkaloids are given on the same day. Our data show that the anti-tumor effect of this drug combination might be enhanced, if both drugs are given on different days.

In vitro, the drug combination was found to be highly sequence-dependent both in tumor cell lines and in primary, patient-derived tumor cells. Sequence dependency was also found in an in vivo trial. Using complex signaling studies and knockdown strategies, we delineate the underlying signaling mechanism for antagonistic apoptosis: Doxo activates p53, p53 induces cell cycle arrest and cell cycle arrest inhibits the downstream apoptosis signaling pathway otherwise activated by VCR. Characterization of this signaling mechanism enabled to identify further antagonistic drug combinations, e.g., irradiation and VCR.

Back in the 1970th, clinicians decided to combine anthracyclines and vinca alkaloids during polychemotherapy based on few (< 10) animal experiments performed on rat and mouse tumor cells (6,7). In these animal trials, high concentrations of doxo potentiated VCR-induced anti-tumor efficiency. Regarding the combination of doxo and VCR and as far as we know, no systematic evaluation of application schedules was ever performed on human tumor cells neither in vitro or in small animal models in vivo nor in clinical trials in patients.

In contrast, in our study we used human leukemia cell lines in vitro and in vivo and primary tumor cells of children with acute leukemia ex vivo and found that anthracyclines markedly inhibited vinca alkaloid-induced apoptosis. Here, independent application of doxo and VCR was more effective against most tumor cells than combined application in vitro. To study the biological effects and signaling mechanisms of chemotherapeutic drugs, we used drug concentrations in vitro which are achieved in the plasma of patients during anti-tumor therapy in vivo (peak plasma concentration = ppc as maximum) (32). The difference between the previous data and our data might rely on factors like inter-species differences, drug concentrations used, different read-outs and the small number of cell lines tested in those former studies.

It will be interesting to evaluate whether the independent application of anthracyclines and vinca alkaloids will increase treatment efficiency in cancer
patients, especially as doxo and VCR showed antagonistic effects in the primary
tumor cells of one third of children treated with this combination.

The newly discovered anti-apoptotic function of doxo is in line with our
previous data showing that TRAIL, a chemotherapeutic drug currently in phase I and
II clinical trials, displays anti-apoptotic and even pro-proliferative features in
apoptosis-resistant tumor cells (18-20).

There is a long-lasting conceptual discussion of whether cytotoxic and
cytostatic drugs should be combined (33). The term 'cytostatic drugs' has been used
for drugs that act against tumor cells by inducing cell cycle arrest. In our study,
anthracyclines arrested the cell cycle and behaved like cytostatic drugs in the cell
lines investigated. On the other hand, several chemotherapeutic drugs require active
cell cycling for induction of apoptosis and cell cycle arrest reduces or abrogates the
anti-tumor efficiency of these drugs (16,33). In our experiments, vinca alkaloids acted
like drugs depending on active cell cycling. Our data allow the hypothesis that
cytostatic drugs and drugs depending on active cell cycling might not be combined,
as they exert optimal anti-tumor efficiency, if given independently.

Vinca alkaloids are not the only class of chemotherapeutic drugs which
depends on active cell cycling (16). More studies are required to identify those drugs,
which share the molecular mechanism of vinca alkaloids and should therefore not be
combined with cytostatic drugs.

p53 was shown by others (34) and by us (19) to predominantly act as a pro-
apoptotic mediator. In contrast to its mainly pro-apoptotic functions, in our study p53
acted as an apoptosis inhibitor towards VCR-induced apoptosis. Although cell lines
contained different p53 conformations and mutations, no correlation was found
between mutations of p53 and interaction between doxo and VCR (22).

The present work clearly underlines the significance to re-evaluate the
inhibitory effect within the multiple known functions of p53 (35). Since many
chemotherapeutic drugs activate p53, the new anti-apoptotic effect might not be
restricted to anthracyclines and irradiation, but might involve additional established
chemotherapeutic drugs that activate p53. Our data allow the hypothesis that further
stimuli of p53 will disable VCR-induced apoptosis.

It is well known that 1) numerous chemotherapeutic drugs activate p53, 2) p53
can induce cell cycle arrest in tumor cells, and 3) certain chemotherapeutic drugs
need active cell cycling for the induction of cell death (16,19,23,29,31). The new anti-
apoptotic function of anthracyclines, although clinically and empirically surprising, is not surprising once the underlying molecular mechanisms are considered.

Our data represent the first characterization of a molecular mechanism responsible for sequence-dependent anti-tumor effects of chemotherapeutic drugs in routine clinical use. Our data clearly reinforce the need for the detailed mechanistic understanding of signaling pathways and pathway cross talks. “Targeted therapies” represent a promising concept to optimize anti-tumor therapy based on the mechanistic understanding of target proteins and cellular signaling. In parallel, our data encourage to widen the concept and search for “targeted drug combinations” in which the positive or negative interaction of drugs is characterized on a molecular level. These new mechanistic insights will enable the design of more effective polychemotherapy protocols to treat hematopoietic malignancies.
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Authorship and Conflict of Interest Statement

Contribution: H.E., D.S., C.M. and F.W. performed experiments; S.H. provided phosphorylation deficient Bcl-xL expression plasmids; U.G. and M.N. provided primary patient samples; H.E. and I.J. designed the research, provided administrative support, analyzed and interpreted the data, prepared the figures and wrote the paper. All authors had a final approval of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


Table 1. New colony formation 7 days after stimulation with doxo and VCR in CEM cells

<table>
<thead>
<tr>
<th>drug</th>
<th>co</th>
<th>doxo</th>
<th>VCR</th>
<th>doxo+VCR</th>
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<tbody>
<tr>
<td>number of colonies</td>
<td>165±15</td>
<td>70±11</td>
<td>2±1</td>
<td>40±4*</td>
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CEM cells were stimulated with doxorubicin (doxo, 30 ng/ml) and / or vincristine (VCR, 30 ng/ml) or left untreated (co) as in Figure 1A using 96 well plates. Consecutive light microscopy pictures were taken for 7 days to detect new colony formation. Drug concentrations were reduced compared to the experimental setting in Figure 1 according to lower cell density. Data are presented as mean of 3 independent experiments ± SEM. *p<0.05, comparing new colony formation after VCR alone to the combined doxo plus VCR treatment.
**Titles and legends to figures**

**Figure 1** Inhibition of vinca alkaloid-induced apoptosis by anthracyclines
A) CEM leukemia cells were simultaneously stimulated with doxorubicin (doxo, 100ng/ml) and vincristine (VCR, 300ng/ml) for time periods indicated. *p < 0.05, ANOVA. NS = not significant.

B) Corresponding data from Figure 1A for 48 hours incubation time and simultaneous application were analyzed for a total of n=20 combinations by median effect plots investigating a range of drug concentrations (doxo 10, 30, 60 and 100ng/ml; VCR 3, 10, 30, 100 and 300ng/ml). fa=apoptotic fraction, fu=fraction of cells alive. d=drug dosage

C-E) Further n=6 B-ALL and T-ALL (C), n=7 acute myeloid leukemia (D) and n=3 lymphoma cell lines (E) were stimulated with doxo and VCR for 48 hours as in Figure 1A. *p < 0.05, ANOVA. NS = not significant.

F) Xenograft study of CEM leukemia cells subcutaneously implanted into NSG mice was performed as described in "Methods". Mice were treated as depicted in the treatment schedule with doxo (0.3 mg/kg) and/or VCR (0.9 mg/kg) or placebo as depicted. Tumor size was measured in two dimensions and tumor volume was calculated. Statistical analysis using Mann-Whitney Rank Sum test was performed comparing VCR and combinatorial treatment (doxo plus VCR) at each measurement point (*p<0.01) and revealed that doxo followed by VCR 1 day later significantly inhibited the effect of VCR alone. Depicted are 25th and 75th quartile; p=placebo, d=doxo, V=VCR.

**Figure 2** Antagonistic effect of doxorubicin and vincristine on primary leukemic tumor cells
A) Three primary leukemia samples (patient # 6, 10 and 12 from Figure 2C) were simultaneously stimulated with doxo (300ng/ml) and VCR (300ng/ml). Apoptosis induction was measured after 48 hours, when spontaneous apoptosis had reached 40%, otherwise after 72 hours.

B) Patient sample #10 (left panel, VCR 300ng/ml) and #6 (right panel, VCR 30ng/ml) from Figure 2A were stimulated with doxo and VCR (as indicated).

C) 35 primary leukemia samples were stimulated with either doxo or VCR alone or simultaneously with doxo and VCR as in Figure 2A. All samples were sensitive
for doxo (specific apoptosis >10%) besides samples 29-32. Measured apoptosis for the combination of doxo and VCR is depicted as black dots (called “simultaneous application”). The expected apoptosis induction, if doxo and VCR were given independently, was calculated from the results obtained with each drug alone as described in “Methods” and is depicted as white and grey bars (called “independent application”). Definition of antagonistic, additive and synergistic apoptosis is described in “Methods”.

Figure 3. Sequence dependent effects of the drug combination doxorubicin / vincristine
A,B) CEM cells were stimulated and analyzed as in Figure 1B, but now doxo was applied 12 hours before VCR (A) or 24 hours after VCR (B).

Figure 4 Signaling mechanism of VCR and inhibition by doxo
A) Western blot of total cellular protein was performed on CEM cells stimulated with doxo and VCR as in Figure 1A. GAPDH served as a loading control.
B) CEM cells were stimulated with doxo and VCR. Loss of mitochondrial membrane potential (left panel) and cytochrome c release (right panel) was measured by FACScan, caspase cleavage was detected by Western blot (lower panel). *p < 0.05, ANOVA, comparing stimulation with VCR alone to doxo alone or combined stimulation with doxo plus VCR.
C) CEM cells were treated with 2,3-DCPE (10 µM, left panel) or the phosphatase inhibitor okadaic acid (okadaic, 0.03 ng/ml, right panel) for 8 hours, followed by doxo together with VCR for another 48 hours as indicated. Western blot of total cellular protein was performed after 56 hours. *p < 0.05, ANOVA. NS = not significant.

The concentrations of doxo and VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A. Casp = Caspase, co = unstimulated control cells, cl. = cleaved, d = doxo, p = phosphorylated, h = hour, V = VCR

Figure 5 Activation of p53 by doxo and its impact on VCR-induced apoptosis
A) Nuclear extracts of CEM cells simultaneously stimulated with doxo and VCR as indicated were analyzed by Western blot. Histone H1 served as a loading control.

B) Parental CEM cells stably transfected with shRNA targeting p53 (shp53) or a control mock shRNA sequence were stimulated with doxo and VCR simultaneously. *p < 0.05, ANOVA. NS = not significant. Western Blot was performed of total cellular protein.

The concentrations of doxo and VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A. d = doxo, V = VCR, h = hour, co = unstimulated control cells

Figure 6 Arrest of the cell cycle by doxo and its impact on VCR-induced apoptosis
A,B) Cell cycle analysis was performed using PI-staining of DNA in CEM cells (A). Corresponding cell cycle histograms are presented in suppl. Figure 3A. To discriminate between G2 and M-arrest, double staining for phospho-Histone H3 (Ser10) and propidium iodide was performed after 24 hours (B). co = unstimulated control cells

C) CEM cells were pre-incubated with caffeine (300 µg/ml) for 8 hours, followed by doxo together with VCR for 48 hours. *p < 0.05, ANOVA. NS = not significant.

D) CEM cells were stably transfected with a shRNA targeting cyclinA (shcyclinA) or a control mock sequence and were analyzed for cell cycle distribution of spontaneously growing cells (left panel) or for apoptosis induction by VCR (3ng/ml) after 48 hours (right panel). *p < 0.05, ANOVA. NS = not significant.

E) Scheme summarizing the data presented in Figures 4, 5 and 6: Doxorubicin-mediated activation of p53 and G2 arrest inhibits VCR-induced cell death, abrogating VCR-induced phosphorylation of Bcl-2 family members, the distal apoptosis signaling pathway and cell death.

The concentrations of doxo and VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A.

Figure 7 Cell cycle arrest-based antagonistic interaction between irradiation and vincristine
CEM cells were irradiated with 6 Gy for 24 hours. Cell cycle analysis was performed (left panel) and cells were stimulated with VCR for another 48 hours (right panel). *p < 0.05, ANOVA. NS = not significant.

The concentration of VCR, measurement of apoptosis, presentation of data, and statistical analysis were performed as described in Figure 1A.
Figure 1

**A**

![Bar chart showing specific apoptosis (%)](chart_a.png)

Specific apoptosis (%) over time for doxorubicin (doxo) and vincristine (VCR) treatments.

**B**

![Logarithmic plot of Log(fa/fu) vs. Log(d)](chart_b.png)

Logarithmic plot showing the relationship between Log(fa/fu) and Log(d) for different treatments.

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Figure 1

E

Specific apoptosis (%)

KMH2          L428           RAJI

lymphoma

doxo          VCR          doxo+VCR

* * * * *

F

Median tumor volume (%)

doxo          doxo+VCR

co

pp pp

dp dp

dV dV

Vp Vp

0 8 15

days

treatment schedule
Figure 2
C

- independent application
- simultaneous application

| patient # | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  | 25  | 26  | 27  | 28  | 29  | 30  | 31  | 32  | 33  | 34  | 35  |
|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----- |

- antagonistic: 12 / 35 = 34%
- additive: 19 / 35 = 54%
- synergistic: 4 / 35 = 11%
Figure 3

A  
- doxo
- VCR
- doxo 12h before VCR (combination)

B  
- doxo
- VCR
- doxo 24h after VCR (combination)
Figure 4

A

B

Casp-1
Casp-2
Casp-8
Casp-10
cl. Casp-3
cl. Casp-6
cl. Casp-7
cl. Casp-9
cl. PARP
GAPDH
Figure 4
Figure 5

A

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B

- Specific apoptosis (%)
  - * indicates statistical significance.

- Histone H1

- p53

- GAPDH

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Figure 6
Figure 6
Figure 6

E

VCR

G2 arrest

p53

shp53

doxo

p53

shcyclinA

KU55933

caffeine

calphostin C

okadaic acid

DCPE

Bcl-2

Bcl-xL

mito

cyt C

caspases

apoptosis
Figure 7
Optimized anti-tumor effects of anthracyclines plus vinca alkaloids using a novel, mechanism-based application schedule

Harald Ehrhardt, David Schrembs, Christian Moritz, Franziska Wachter, Subrata Haldar, Ulrike Graubner, Michaela Nathrath and Irmela Jeremias