# Pharmacokinetic and cell biological aspects of vincristine efficacy in childhood leukaemia

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**Summary**

Vincristine, one of the naturally occurring vinca alkaloids extracted from the leaves of the periwinkle plant *Catharanthus roseus*, remains to be an important anti-neoplastic drug in paediatric oncology since its introduction in the early nineteen sixties. Vincristine is active against acute leukaemia, Hodgkin's and non-Hodgkin's lymphoma, soft tissue sarcoma, Wilm's tumour, brain tumour and neuroblastoma. The dose-limiting side effect consists of a peripheral sensory, motor, and autonomic neuropathy. Other side effects, such as bone-marrow suppression, are relatively mild and no serious long-term side effects are known. Because of its wide applicability and relatively mild bone-marrow suppression, vincristine is easy to combine with other anti-neoplastic drugs in a combination chemotherapy regimen. Therefore, it is worthwhile to try to optimize the treatment with vincristine in children.

Two goals can be distinguished when trying to optimize vincristine treatment: suppression of side effects and improvement of efficacy. The research presented in this thesis was focussed on improvement of vincristine efficacy. The antileukaemic effect of vincristine is principally determined by two factors: systemic exposure to vincristine and *in vivo* cell sensitivity to vincristine. First we hypothesized that the antileukaemic effect of vincristine might vary between patients as a consequence of interindividual variability in vincristine systemic exposure. If antileukaemic effect and systemic exposure are correlated, we could try to improve response by pharmacokinetically guided adaptation of the vincristine dose in each patient. The second part of the thesis was focussed on the mechanism of action of vincristine since better knowledge of its mechanism of action might help to understand differences in cell sensitivity to vincristine.

To place vincristine in a broad perspective of cancer chemotherapy in children, in **chapter 1** we reviewed the current knowledge of pharmacokinetics and –dynamics of some anti-neoplastic drugs, used in the treatment of childhood

cancer, and the experience with pharmacokinetically guided, individualized dosing. Of all reviewed anti-neoplastic drugs, methotrexate appears to be most extensively studied. Methotrexate pharmacokinetics is correlated with toxicity and response to therapy, and individualized adaptive dosing of methotrexate is correlated with a better response to therapy, without increasing toxicity, in children with acute lymphoblastic leukaemia and osteosarcoma. For most of the other reviewed anti-neoplastic drugs, including vincristine, it is demonstrated that pharmacokinetics is correlated with toxicity. Of some drugs a relationship of pharmacokinetics with response to therapy is demonstrated as well. In case of cytarabine, etoposide, and teniposide, individualized dosing also appears to be feasible. However, there is no evidence that this strategy improves response to therapy. In children with acute lymphoblastic leukaemia and/or acute myeloid leukaemia, cytarabine and teniposide adaptive dosing did not improve response. The effect of etoposide adaptive dosing on toxicity and response to therapy has not been studied up till now. Future research might answer the question whether this strategy improves etoposide response, and whether pharmacokinetics of carboplatin, ifosfamide, paclitaxel, and vincristine are correlated with response in children. There is a lack of knowledge on pharmacokinetic and –dynamic correlations in children for the anthracyclines, bleomycin, cisplatin, cyclophosphamide, and vinblastine. The research presented in this thesis, addresses the question whether pharmacokinetics of vincristine is correlated with response to vincristine monotherapy.

In the review, we also summarized existing knowledge from previous studies on vincristine pharmacokinetics and –dynamics. Vincristine is usually administered as an intravenous bolus dose and its disposition is described by a two-compartment, first-order model. Mean (SD) terminal elimination half-life and systemic clearance in children varied from 13.7 (6.5) to 18.7 (18.8) hours and 357 (146) to 482 (342) ml/min/$m^2$ respectively, in different reports. In adult lung cancer patients, mean vincristine clearance was approximately 190 ml/min/$m^2$. Intra- and

interindividual variability of pharmacokinetic parameters in children appeared to be large.

Renal elimination of vincristine accounts for approximately 10% of systemic clearance in adults. Vincristine is predominantly excreted by the hepatobiliary pathway and a considerable amount of the parent drug is subject to hepatic metabolism before excretion into the bile. In line with the important role of hepatic clearance in vincristine elimination, increased serum alkaline phosphatase concentrations were associated with increased systemic exposure and elimination half-life of vincristine in a group of adult cancer patients, suggesting impaired hepatobiliary elimination. In several case reports severe vincristine toxicity was associated with co-administration of CYP3A4 inhibiting drugs during vincristine treatment and in some studies vincristine pharmacokinetics appeared to be affected by these drugs.

In each of the previous pharmacokinetic studies of vincristine in children, vincristine was administered in combination with other cytotoxic drugs or corticosteroids. Drug interactions may have contributed to the large interindividual variability that was detected in these studies. In **chapter 2**, we described the pharmacokinetics of vincristine as a monotherapy in a group of 70 children newly diagnosed with acute lymphoblastic leukaemia. Thus, these patients did not receive corticosteroids or other cytotoxic drugs during the period of the study. Vincristine plasma concentrations were measured by HPLC analysis, after a single dose of 1.5 $mg/m^2$ vincristine i.v. The data were fitted according to a two-compartment, first-order pharmacokinetic model by maximum *a posteriori* parameter estimation and secondary pharmacokinetic parameters were calculated from the model. In this relatively homogeneous group of children pharmacokinetic parameters were highly variable: median ($25^{th}$ and $75^{th}$ percentiles) total body clearance, 228 (128 – 360) $ml/min \cdot m^2$; elimination half-life, 1001 (737 – 1325) min; apparent volume of distribution at steady state, 262 (158 – 469) $L/m^2$. Variability of vincristine pharmaco-

kinetics remains largely unexplained despite examination of the influence of several demographic and biochemical variables. However, vincristine clearance is substantially slower than has been reported previously for children receiving vincristine in combination with steroids as part of combination chemotherapy (median clearance, 228 ml/min·$m^2$ versus mean clearance, 381 and 482 ml/min·$m^2$, respectively). Steroids are known as inducers of vincristine-metabolizing CYP3A4 enzymes. The absence of steroids during our study appears to be the most likely explanation for this difference.

In **chapter 3** we addressed the question whether pharmacokinetics of vincristine is correlated with response to vincristine monotherapy. In the above mentioned group of 70 children newly diagnosed with acute lymphoblastic leukaemia, not only pharmacokinetic parameters were studied but response to vincristine monotherapy was studied as well. Before and three days after administration of a bolus dose of 1.5 mg/$m^2$ vincristine, the percentage of lymphoblasts was determined on bone marrow and peripheral blood smears. In addition, the white blood cell count was determined, which allowed evaluation of three response parameters: a decrease of the percentage of lymphoblasts in bone marrow and peripheral blood, and a decrease of the absolute number of lymphoblasts in peripheral blood. The combination of at least one response parameter and vincristine pharmacokinetics was available in 54 patients. Results of the study in chapter 3 show that variability of response to vincristine monotherapy, in bone marrow and peripheral blood, could not be attributed to interindividual variability of vincristine systemic exposure. The lack of pharmacokinetic and – dynamic correlations, demonstrated in this study, does not support a role for therapeutic drug monitoring of vincristine in children with acute lymphoblastic leukaemia.

Our second approach to improve vincristine efficacy was focussed on vincristine's mechanism of action, since an important step to better efficacy of a

drug is an improved knowledge of its mechanism of action. The antileukaemic effect of vincristine is traditionally attributed to inhibition of cell cycle progression due to interference with microtubule dynamics. However, it was also shown *in vitro* that vincristine induces apoptosis in leukaemic cell lines. Apoptosis is a process of cell death characterized by nuclear fragmentation with double-stranded DNA breaks, and cytoplasmic condensation and formation of apoptotic bodies, which can be removed by phagocytosis. The investigational up-front window study, described in this thesis, provided a unique opportunity to study vincristine-induced apoptosis *in vivo* in children with acute lymphoblastic leukaemia.

The results described in **chapter 4**, demonstrate that vincristine induces apoptosis *in vivo* in peripheral blood mononuclear cells, mainly lymphoblasts, of children with acute lymphoblastic leukaemia. In five patients, participating in the investigational up-front window study of DCLSG protocol ALL-9, extra peripheral blood samples were collected on the first day of the study. The sampling was scheduled before and 3, 8, 12, 16, 20, and 24 h after vincristine administration. Peripheral blood mononuclear cells were isolated by density gradient centrifugation of the blood on Ficoll-Paque. The TUNEL (*terminal deoxy-nucleotide transferase-mediated dUTP-digoxigenin nick-end labelling*) assay was performed on 4% paraformaldehyde-fixed cytospin slides of mononuclear cells to detect apoptosis. In one patient, a striking increase of apoptotic cells from 2% (SEM 0.6) before to 40.7% (SEM 2.9) 3 h after vincristine injection was found. In the other patients the maximum increase ranged from 0.8% to 4.3%. Wilcoxon matched pairs analysis of all patients confirmed that vincristine induces apoptosis *in vivo* in peripheral blood mononuclear cells in children with acute lymphoblastic leukaemia.

In **chapter 5** we further studied the route of vincristine-induced apoptosis. In the process of apoptosis caspases play a critical role. Caspases are cysteine proteases that exist as proenzymes and are activated upon cleavage. In cell-free

extracts activation occurs in a hierarchical order. Evidence is accumulating that the caspase cascades differ depending on the apoptotic stimulus. In apoptosis induced by CD95-receptor signalling, caspase-8 is the most proximal caspase, activated by autoproteolysis after recruitment to the intracellular located adaptor proteins FADD of the CD95-receptor complex. In apoptosis induced by apoptotic stimuli that activate the mitochondrial controlled pathway, the most proximal caspase is caspase-9, which can be activated after cytochrome C and dATP-dependent formation of a caspase-9/Apaf-1 complex. It is shown that mitochondrial changes such as cytochrome C release, loss of mitochondrial membrane potential and the production of reactive oxygen species, can precede caspase-9 activation during apoptosis. Furthermore, it is known that the pro- and anti-apoptotic members of the Bax/Bcl-2 protein family regulate mitochondrial cytochrome C release and apoptosis. Apoptosis induced by several DNA-damaging anti-neoplastic drugs does not require CD95-receptor signalling but most often follows a CD95-independent, mitochondrial controlled pathway. It is unclear whether apoptosis induced by vincristine proceeds by a mitochondrial controlled pathway.

The results presented in **chapter 5** demonstrate caspase-9 and -3 activation *in vivo* in bone marrow leukaemic cells of a child with newly diagnosed acute lymphoblastic leukaemia, after treatment with a single dose of vincristine. We hypothesized that vincristine induces apoptosis by a mitochondrial controlled pathway and we further studied its mechanism of action in Jurkat acute lymphoblastic leukaemia cells. Vincristine-induced activation of caspase-9 and subsequently caspase-3 was demonstrated by western blot analysis of cell lysates, prepared after exposing Jurkat cells to vincristine with or without the caspase-9 inhibitor Z-LEHD-FMK. Loss of mitochondrial transmembrane potential was independent of caspase-9 activation. To confirm the mitochondrial role in vincristine-induced apoptosis, the effect of blocking the mitochondrial route upstream of caspase-9 activation was investigated at two different levels: reactive

oxygen species scavenging and Bcl-2 overexpression. We found that generation of reactive oxygen species was detected early in Jurkat cells during vincristine exposure. Ascorbic acid, a reactive oxygen species scavenger, inhibited reactive oxygen species generation as well as caspase-9 and -3 activation and cell death induced by vincristine. Furthermore, in Bcl-2 overexpressing Jurkat cells mitochondrial transmembrane potential changes, caspase-9 and -3 activation, and cell death upon vincristine exposure were decreased, in comparison to parental Jurkat cells. However, generation of reactive oxygen species was not decreased in Jurkat cells with Bcl-2 overexpression. We concluded that reactive oxygen species play a regulatory role in the initial phase of a mitochondrial controlled pathway of vincristine-induced apoptosis in acute lymphoblastic leukaemia cells.

We realized that the antileukaemic effect of vincristine *in vivo* might be impaired by leukaemic cell resistance to apoptosis, despite adequate intracellular drug concentrations. Therefore, it would be useful to determine intracellular concentrations of vincristine in leukaemic cells of patients. In **chapter 6** we described a high-performance liquid chromatographic (HPLC) method to measure vincristine concentrations in human mononuclear cells. This method with on-line column, solid-phase extraction and electrochemical detection, previously developed for determination of vincristine concentrations in human plasma, was validated for determination of intracellular vincristine concentrations over a range of 1.17 to 50.8 μg/L with a lower limit of quantitation of 1.17 μg/L. The method was used successfully to measure intracellular vincristine concentrations in 8/35 bone marrow mononuclear cell samples of children newly diagnosed with acute lymphoblastic leukaemia, three days after the first injection of 1.5 $mg/m^2$ vincristine. Vincristine concentrations were in the range of 4.0 to 26.4 μg/L which was 5 to 20 times the bone marrow plasma concentration.

We conclude that vincristine pharmacokinetic parameters are highly variable, even in a relatively homogeneous group of children newly diagnosed with ALL,

and that this variability remains largely unexplained. Vincristine clearance is substantially slower after vincristine monotherapy than has been reported previously for children receiving vincristine as part of combination chemotherapy, which is most likely due to the absence of steroids, inducers of vincristine-metabolizing cytochrome $P_{450}$ 3A4 (CYP3A4) enzymes, in our study. Variability of response to vincristine monotherapy could not be attributed to interindividual variability of vincristine systemic exposure. Induction of apoptosis appeared to be an important mechanism of antileukaemic action of vincristine *in vivo* in mononuclear cells of children newly diagnosed with acute lymphoblastic leukaemia. Reactive oxygen species play a regulatory role in the initial phase of a mitochondrial controlled pathway of vincristine-induced apoptosis in Jurkat acute lymphoblastic leukaemia cells.

Results of the research presented in this thesis suggest that activity of vincristine-metabolizing CYP3A4 enzymes is an important determinant of vincristine systemic exposure. Polymorphisms of the genes coding for CYP3A4 enzymes might be responsible for part of the variability of vincristine systemic exposure. Studies are presently undertaken to determine CYP3A4 polymorphisms and its relationship with vincristine pharmacokinetics. Other polymorphisms, e.g. in the genes coding for drug-efflux pumps, might also play a role. The lack of pharmacokinetic and –dynamic correlations, demonstrated in this thesis, does not support a role for individualized adaptive dosing of vincristine in children with acute lymphoblastic leukaemia at this moment. It is necessary to further unravel the route of vincristine-induced apoptosis in acute lymphoblastic leukaemia cells and the role of reactive oxygen species in this pathway. Variability between different cell lineages in the ability to eliminate reactive oxygen species might be responsible for differences in cell sensitivity to vincristine.