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SHORT COMMUNICATION

Impact of plasma and intracellular exposure and *CYP3A4*, *CYP3A5,* and *ABCB1* genetic polymorphisms on vincristine-induced neurotoxicity

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Abstract
Purpose The aim of this study was to investigate the impact of plasma and intracellular exposure and *CYP3A4*, *CYP3A5,* and *ABCB1* polymorphisms on vincristine neurotoxicity. We subsequently assessed the impact of *ABCB1* polymorphisms on intracellular vincristine accumulation.
Methods Children treated for solid tumors were enrolled in the study ($n = 26$) and received 1.5 mg/m^2 of vincristine per course. Individual pharmacokinetic parameters and *CYP3A4*, *CYP3A5,* and *ABCB1* genotypes were available from a previous analysis. A global toxicity score (pain, peripheral neurotoxicity, and gastrointestinal toxicity) was collected at each course. Vincristine in plasma and PBMCs were quantified by LC-MS/MS.
Results Vincristine plasma and intracellular concentrations ranged from 0.40 to 89.6 ng/ml and from 0.00225 to 1.85 ng/10^6 cells over a 24-h interval, respectively. The global toxicity score ranged from 0 to 6 and was not correlated with individual pharmacokinetics parameters. Neurotoxicity events (global score $\geq$3) were observed in 8 patients but the incidence was not influenced by the different studied polymorphisms. The global toxicity score was correlated with age, body surface area, and dose in mg. A trend to higher intracellular/plasma ratio of vincristine was found for patients with heterozygous diplotype (CGC-TTT) of *ABCB1*.
Conclusions None of the different genetic covariates nor plasma and intracellular exposure was predictive of the observed neurotoxicity in our pediatric population. Nevertheless, the heterozygote diplotype of *ABCB1* appears to influence the intracellular accumulation of vincristine. Owing to the small sample size, further evaluations are needed in a larger patient cohort.

Keywords Vincristine · Neurotoxicity · Pediatric · Pharmacogenetics · Pharmacokinetics · Intracellular concentration

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Introduction

Vincristine has been used for more than four decades to treat children with acute lymphoblastic leukemia (ALL) and is currently included in many protocols for pediatric solid tumor diseases. The most frequent and clinically relevant side effect of vincristine is neurotoxicity, and no specific preventive and/or curative treatment is available to manage this toxicity. The only current recommendation is to reduce vincristine doses or discontinue vincristine treatment, which may impair treatment efficacy. Moreover, it is commonly accepted that the vincristine dose must be capped at 2 mg per administration.

The reported factors associated with vincristine-induced neurotoxicity include the pre-existence of some neurological afflictions, an age more than 60 years, an association with neurotoxic drugs, alcoholism, and diabetes [1, 2]. Severe neuropathies have been observed in patients with hepatic failure [3, 4] and drug–drug interactions with itraconazole and nifedipine have been reported to lead to an increased risk of neurotoxicity [5]. Thus, higher concentrations of vincristine, through cytochrome P450 3A (CYP3A) inhibition or a hepatic dysfunction, could increase the risk of neurotoxicity. In 1994, Crom et al. [6] found no association between neurotoxicity and vincristine systemic exposure. To date, no study has described the pharmacokinetics parameters of vincristine as risk factors for neurotoxicity.

A genetic implication has also been suggested to explain vincristine toxicity. Indeed, vincristine is metabolized through the CYP450 system, mainly by the CYP3A4 and CYP3A5 isoforms, and partly transported by the P-glycoprotein (P-gp, encoded by the *ABCB1* gene). Several single-nucleotide polymorphisms (SNPs) have been described on *CYP3A4*, *CYP3A5,* and *ABCB1* genes [7]. Both the *CYP3A*3* and *CYP3A4*1B* allelic variants have been associated with a decrease in or an absence of CYP3A5 and CYP3A4 activity, respectively. The genetic hypothesis has been enhanced by the work of Renbarger et al. [8] who reported an effect of race on vincristine-associated neurotoxicity in pediatric ALL patients. More recently, an increase in vincristine neurotoxicity in *CYP3A5* non-expressers has been observed [9], and the severity of neuropathy was inversely correlated with concentrations of M1 (vincristine metabolite) but not of vincristine. Nevertheless, we previously reported no impact of *CYP3A4*, *CYP3A5,* and *ABCB1* polymorphisms on the vincristine pharmacokinetics in a pediatric population treated for solid tumors [10].

Elsewhere, in vitro studies have demonstrated that vincristine accumulates in cells during exposure interval [11, 12]. In vivo intracellular concentrations of vincristine in bone marrow mononuclear cells 5–20 times higher than those in plasma were observed 3 days after vincristine administration [13]. These results suggest that vincristine toxicity could also depend on intracellular accumulation of vincristine. Intracellular accumulation of vincristine is partly mediated by the P-gp [14]. Consequently, it would be also interesting to evaluate the relationship between intracellular vincristine concentration and *ABCB1* polymorphisms.

In this context, we decided to perform a pilot study to investigate the relationship between vincristine-induced neurotoxicity and both plasma and intracellular vincristine concentrations and *CYP3A4*, *CYP3A5,* and *ABCB1* polymorphisms. We also assessed the impact of *ABCB1* polymorphisms on intracellular vincristine accumulation.

Methods

Patients

Patients included were the same patients who participated in a precedent pharmacokinetic-pharmacogenetic study [10]. Therefore, pharmacokinetic parameters and *CYP3A4* and *CYP3A5* genotypes and *ABCB1* diplotypes were already available [10]. This study was approved by the Ethics Committee of the Pasteur University Hospital (Nice, France), and enrollment of patients took place after written informed consent. Twenty-six children, treated according to the protocol in use for their tumor, were included in this analysis. They were followed up over three courses of vincristine that were not necessarily consecutive. Patients received 1.5 mg/m^2 of vincristine as an i.v. bolus injection once a week, for all the treatment protocols. The maximum vincristine dose was capped at 2 mg per course. The vincristine dose could be reduced by the patient's physician in case of vincristine-induced neurotoxicity, whatever the toxicity grade. Blood samples were drawn following a sparse sample strategy over 3 courses, as previously described [10].

Evaluation of toxicity

Toxicity data were prospectively collected as part of clinical care on each course and evaluated using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 (available at http://www.ctep.cancer.gov/protocolDevelopment). In terms of vincristine-induced neurotoxicity, the different data collected were pain, peripheral neurotoxicity, and gastrointestinal toxicity (graded from 0 to 4). The 3 grades obtained for each neurotoxicity item were then added to present a global toxicity score (ranged from 0 to 12). A global toxicity score $\geq$3 was considered clinically relevant for detecting at least a mild neuropathy and thereby defined as a neurotoxicity event (NE).

Quantification of vincristine in plasma and peripheral blood mononuclear cell

PBMC were isolated from plasma by density gradient separation with lymphocyte separation medium reagent (Eurobio, France), washed twice and stored at −80°C. Then, extraction of intracellular vincristine was carried out according to the method described by Groninger et al. [13]. Vincristine in plasma and cellular supernatants was quantified by a validated LC-MS/MS method [15]. Chromatographic and spectrometric conditions were similar to those used to quantify vincristine in plasma. A specific matrix was used for the calibration curve (10% human plasma in

PBS) as already described [13]. This method has been validated with a limit of quantification of 0.25 ng/ml and a calibration curve linear up to 50.0 ng/ml.

Determination of vincristine intracellular concentrations

After the extraction step, protein concentration in supernatant was quantified by the Bradford method using Biorad Protein Assay Reagent (Biorad life Science, Munich, Germany). Intracellular concentration of vincristine in ng/mg of protein was then converted into ng per millions of cells, using a linear regression representing the protein concentration according to the number of cells ($y = 1E-06x + 0.27$, $r^2 = 0.97$), built with blood samples from the same pediatric population for which cell counting was available.

To present the intracellular/plasma concentration ratio, we estimated the intracellular vincristine concentration in ng/ml, taking into account that the volume of 1×10^6 cells is 400 µl [16]. The intracellular/plasma vincristine ratio were compared with both the incidence of neurotoxicity events and the main diplotypes of *ABCB1*.

Data analysis

Statistical analysis was performed using Sigmastat version 2.03 software (SSPS Inc., San Rafael, USA). Linear regression test was used to study the independent effect of clearance (CL), intercompartimental clearance (Q), terminal half-life (T1/2), area under the curve ($AUC_{0\rightarrow24}$), age, body area, dose, dose per mg, and cumulative dose on global toxicity score. The incidence of neurotoxicity events was compared between *CYP3A4*, *CYP3A5,* and *ABCB1* SNPs using the χ^2 test. The Mann–Whitney *U* test was used to analyze the differences in intracellular/plasma vincristine ratio between the main *ABCB1* diplotypes and the frequency of neurotoxicity events. An intergroup difference was considered as statistically significant for a *P* value <0.05.

Results

A total of 26 children were included in the study (15 M/11F). The cohort was composed of Caucasian-origin patients only, aged from 2 to 16 years (8.8 ± 4.2 years). Mean body surface area and weight were, respectively, 0.94 ± 0.34 m^2 and 27.5 ± 15.4 kg. Patients were treated for various solid tumor diseases as already described [10]. None of the patients had evidence of pre-existing neuropathy, liver dysfunction, or diabetes, and no co-medications were known to interfere with the pharmacokinetics of vincristine. A mean dose of 1.33 ± 0.28 mg/m^2 of vincristine was administered. Due to vincristine-induced neurotoxicity, the dose was 50% reduced in two patients, and vincristine was discontinued in five cases. The mean (SD) cumulative vincristine dose at time of enrollment on the study was 7.35 (5.30) mg/m^2.

Only 67 courses were available for pharmacokinetic analysis, corresponding to 184 blood samples. Intracellular concentrations were determined for only 65 samples (16 patients) because blood volume was not always sufficient for PBMC isolation. Vincristine plasma concentrations ranged from 0.40 to 89.6 ng/ml, and intracellular concentrations ranged from 0.00225 to 1.85 ng/10^6cells. Figure 1a shows the observed vincristine concentrations in PBMC versus time.

Toxicity data were available for 65/67 courses in 24 patients, and the global toxicity score ranged from 0 to 6 (number of courses): 0 ($n = 17$); 1 ($n = 21$); 2 ($n = 14$); 3 ($n = 9$); 4 ($n = 1$); 5 ($n = 2$); 6 ($n = 1$). No toxicity grades 3 or 4 were observed in each score separately. A NE (global score ≥3) was observed in 13 courses, i.e., 8 patients (33%).

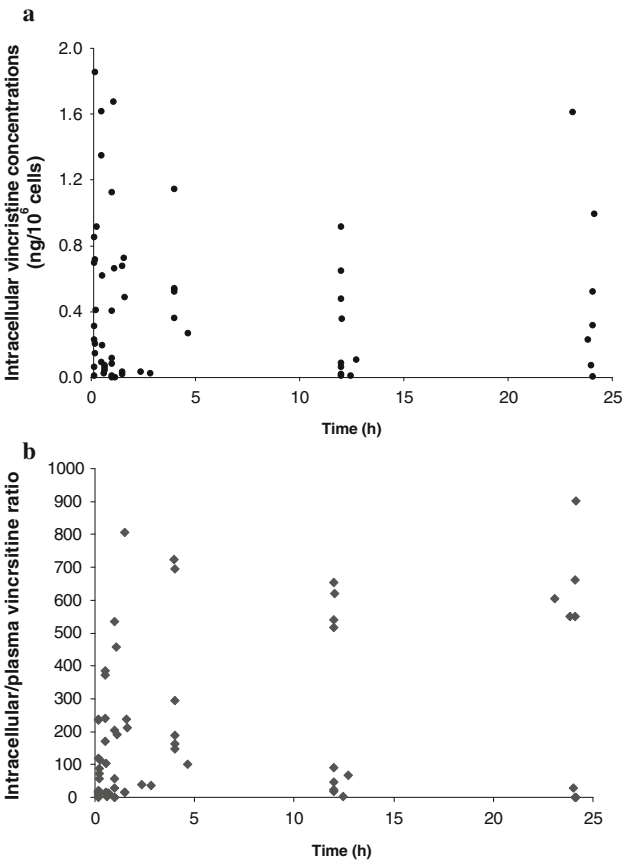

Fig. 1 Observed vincristine concentrations in PBMC **a** or intracellular/plasma vincristine concentration ratio **b** versus time

No difference was observed between the incidence of neurotoxicity events and both *CYP3A4* ($P = 1.00$) and CYP*3A5* genotypes ($P = 0.72$) and *ABCB1* haplotypes ($P = 0.62$). Individual pharmacokinetics parameters did not have an impact on global score of toxicity (CL, $P = 0.12$; Q, $P = 0.40$; T1/2, $P = 0.11$; $AUC_{0\rightarrow24}$, $P = 0.84$). A positive linear regression was found between the global toxicity score and age ($P = 0.002$, $r^2 = 0.34$), body surface area ($P = 0.006$, $r^2 = 0.28$) and the dose in mg ($P = 0.006$, $r^2 = 0.29$) contrary to the dose per m^2 ($P = 0.15$) and the cumulative dose ($P = 0.40$).

Intracellular/plasma vincristine ratio ranged from 0.50 to 902 over a 24-h period post-vincristine administration (Fig. 1b) and was not correlated with the incidence of neurotoxicity events (Table 1). Among the three main diplotypes of *ABCB1*, we did not observe a significant difference in the intracellular/plasma ratio. When we compared patients carrying the heterozygous diplotype (CGC-TTT) with those carrying the wild-type diplotype CGC-CGC or the CGC-CGT, a trend to higher intracellular/plasma ratio of vincristine was found but only at 10 and 30 min post-dose (Table 1).

Discussion

Vincristine neurotoxicity is characterized by a great inter-patient variability that could have a significant impact on therapeutic success. Clinical observations have reported a correlation between an increase in neurotoxicity and a decrease in vincristine metabolism [4, 5]. However, pharmacokinetic data were lacking to confirm this hypothesis. In our population of children treated for solid tumor diseases, individual pharmacokinetic parameters did not have any impact on the global toxicity score. Such results suggest that vincristine pharmacokinetic parameters are not predictive of the neurotoxicity and are in accordance with a precedent study on 54 children treated for ALL [6]. Contradictory results have been recently presented in a large cohort of 107 patients [9]. The authors observed that dose normalized M1 plasma concentrations, 1 h after vincristine administration, were inversely related to the severity of induced neuropathy, suggesting that a decrease in vincristine metabolism could be related to neurotoxicity. But the evaluation of such a relationship using only a 1-h post-dose concentration seems to be inadequate in order to assess vincristine metabolism. Indeed, a higher concentration of vincristine would be expected in such case and was not found in this work. In summary, vincristine plasma pharmacokinetics and neurotoxicity are not clearly correlated. Owing to the high variability of vincristine pharmacokinetics, sample size, low toxicity score, and heterogeneity in treatment regimens, it is difficult to confirm the total

Table 1 Intracellular/plasma ratio of vincristine according to neurotoxicity events and *ABCB1* diplotypes

Intracellular/plasma vincristine concentrations ratio*														
	Time post-administration of vincristine													
	n	10 min	*n*	30 min	*n*	60 min	*n*	90 min	*n*	240 min	*n*	720 min	*n*	1,440 min
Neurotoxicity														
NE	4	80.8 (62.0)	2	170; 9.14	4	243 (491)	1	16.0	2	163; 696	2	90.3; 45.3	3	549 (550)
No NE	10	18.9 (119)	9	15.3 (273)	4	124 (175)	6	126 (251)	5	189 (306)	8	291 (566)	5	552 (683)
P value		0.37		0.91		0.89		–		0.86		0.89		0.79
ABCB1 diplotypes														
CGC-CGC														
CGC-CGC	7	19.2 (66.6)	4	12.6 (4.01)	3	55.9 (423)	5	212 (289)	2	101; 295	4	302 (548)	1	549
CGC-TTT	4	175 (178)	3	102 (293)	2	204; 457	–	–	2	147; 189	2	3.51; 22.5	4	606 (571)
P value		0.07		0.057		0.80		–		1.00		0.27		

* Results are presented as median (interquartile range) or as value(s) if *n* was <3

absence of a correlation in our study and further assessments would be required in a larger population. However, it is quite difficult to enroll children in pharmacokinetic studies, which justified our choice of a population pharmacokinetic study, aiming at both decreasing the number of samples and the blood volume taken.

In the study by Egbelakin et al. [9], the authors observed a greater increase in vincristine neuropathies in patients carrying the *CYP3A5*3/*3* genotype than in those with the *CYP3A5*3/*1* genotype. These results confirm two previous studies where an impact of racial differences on vincristine-associated neurotoxicity was reported [8, 17]. In our study, no link was found between the *ABCB1*, *CYP3A4,* and *CYP3A5* polymorphisms and the incidence of neurotoxicity, defined by the frequency of neurotoxicity events. Nevertheless, the low allele frequencies of the SNPs observed in our small population does not allow us to give definitive conclusions. It is well known that genetic studies require a large number of patients but our choice was based on a very pronounced expected effect according to the literature data [4, 5]. Furthers studies are needed with a larger patient cohort, including different races. The differences observed in the vincristine-induced neuropathy could be also due to genetic polymorphisms affecting sensitivity to vincristine. The microtubule cytoskeleton is the main target of vincristine and is composed of several forms of alpha and beta-isotypes and microtubule-associated proteins (MAPs) which can have an impact on sensitivity to anti-tubulin agents [18]. Part of the key to deciphering vincristine neurotoxicity could therefore lie in the tubulin isotypes and MAP composition within targeted axons.

Owing to the marked intracellular accumulation of vincristine, one other hypothesis would be that neurotoxicity may depend on intracellular exposure rather than plasma exposure. Significant variability was observed in intracellular concentrations and in the intracellular/plasma ratio (0.50–902), whatever the time of sampling. Such results confirmed the large and variable accumulation of vincristine in mononuclear cells. This variability was not correlated with neurotoxicity regardless of the time of sampling in our study. However, Fig. 1a shows that intracellular vincristine concentration does not decrease over the time studied, suggesting a long intracellular half-life. Therefore, it would be more accurate to evaluate the relationship with neurotoxicity on a later time post-dose. Finally, vincristine in PBMC is possibly not a good marker of the risk of neurotoxicity because does not reflect accurately the concentration in neurons.

Nevertheless, it would appear that two populations emerge according to the intracellular/plasma concentration ratio (Fig. 1b). This dichotomic distribution could be due to variations in vincristine transport through the P-gp. This hypothesis is enhanced by a recent study where an increase in vincristine brain tumor concentration was observed in P-gp knock-out mice [19]. The main *ABCB1* diplotypes identified in our population did not display any significant impact on the accumulation ratio. However, a tendency to a greater accumulation of intracellular vincristine was observed in patients with CGC-TTT diplotypes in the early post-dose period, corresponding to the distribution phase of the drug. Such findings emphasize the hypothesis of an influence of the CGC-TTT diplotype on vincristine efflux and intracellular accumulation. These results have to be confirmed on a larger cohort, as well as the impact of other transporters, which might play a role in the distribution of vincristine.

We observed that neurotoxicity was positively correlated with dose per course, age, and body surface area. Similar observations on age and body surface area have been recently reported in 4,567 patients [20]. The increase in the dose per course, which is calculated using the body surface area, proportional to age in children may explain our observation. These results would confirm the interest of the capped dose of 2 mg per course, which is currently recommended. However, additional pharmacokinetic-pharmacodynamic data are needed to confirm this practice.

In conclusion, vincristine plasma and intracellular exposure do not seem to be predictive of the observed neurotoxicity in our pediatric population treated for solid tumors. The findings on the impact of SNPs studied are difficult to make because of lack of power of our study and the low allele frequencies observed. However, some patients seem to present a greater intracellular accumulation of vincristine, which may be related to *ABCB1* polymorphisms. The clinical significance and relevance of such an association remain to be explored in a larger cohort. Finally, a modification in vincristine sensitivity related to variability in tubulin isotypes or MAPs should also be explored.

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