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Chapter 1

Vincristine as an Inductor of Drug Resistance Marker Expression in Neoplastic Cells

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Abstract

Vincristine is known as an effective agent for chemotherapy of neoplastic diseases. Its main pharmacological activity is known to be linked to its inhibition of microtubule dynamics, leading to mitotic arrest and eventual cell death. In particular, vincristine and other vinca alkaloids destabilize microtubules by the induction of microtubule

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depolymerization and mitotic spindle destruction. However, the effectiveness of vinca alkaloids in chemotherapy may also be associated with their influence on other mechanisms, such as the induction of apoptosis through the modulation of pathways related to p53 and p21. Both p53 and p21 are also involved in the regulation of several protein kinase phosphorylation cascades that have significant impact on several cellular functions. These activities determine the effectiveness of vincristine as a chemotherapeutic agent that depresses cell proliferation, induces apoptosis of neoplastic cells, and consequently acts against neoplastic disease progression.

Unfortunately, this drug is also known to induce some undesirable events that alter the sensitivity of neoplastic cells towards different cytotoxic agents. A lack of cell sensitivity to chemotherapy after consecutive treatments with vincristine may occur and is induced *via* several phenotypic changes in the expression of specific proteins including: drug transporters of the ABC family; drug modifying enzymes such as members of the cytochrome P450 and glutathione S-transferase family; proteins involved in the regulation of apoptosis progression such as members of the Bcl-2 protein family; protein kinases involved in the mechanisms of cellular response to chemical stress, such as mitogen-activated protein kinases; and many others. These changes induced by vincristine treatment could be mediated through transcriptional control of nuclear receptors, of which pregnane X receptors seem to play a central role. As a consequence of these changes, vincristine treatment may lead to the development of cell resistance to large groups of chemotherapeutic agents (multidrug resistance), which can lead to a poor prognosis for the consecutive disease treatment of particular patients. Prevention of multidrug resistance development during chemotherapy of neoplastic diseases with vincristine and finding effective chemotherapeutic tools for the treatment of multidrug resistant neoplastic tissues are crucial tasks needed for the improvement of cancer chemotherapy by these drugs. Knowledge about the mechanisms of multidrug resistance development will play an essential role in achieving the latter goal. The aim of this current contribution is to bring state of the art information about these topics to the scientific community.

Keywords: Vincristine, multidrug resistance, ABC transporters, drug resistance markers

Vincristine (VCR), a vinca alkaloid from *Catharanthus roseus*, is generally known as a mitotic inhibitor with a corresponding application in cancer chemotherapy. It is formed *via* the coupling of the indole alkaloids vindoline and catharanthine in the vinca plant (Figure 1) [1].

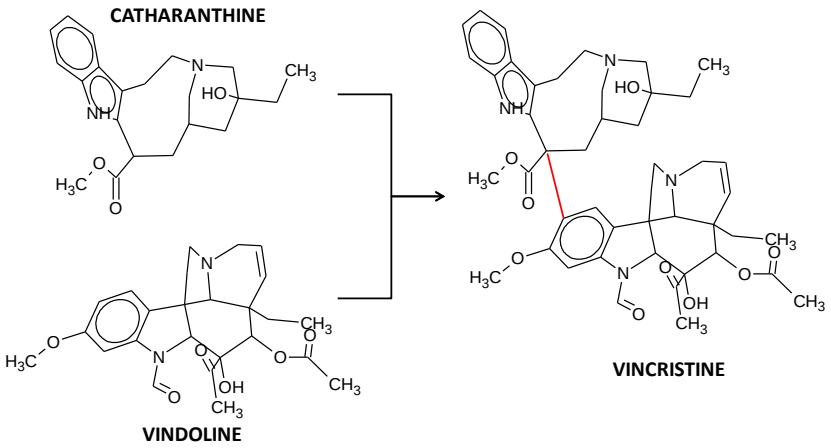

Figure 1. Structure of vincristine. Vincristine is formed *via* the coupling of the indole alkaloids vindoline and catharanthine in the vinca plant. Structures were drawn by ACD/ChemSketch freeware software.

Vincristine is known to block the proliferation of rapidly dividing cell types, including neoplastic cells, *via* interaction with tubulin and inhibition of microtubule assembly [2]. Vinca alkaloids induce alterations in the formation and functions of the mitotic spindle that induce mitotic arrest and inhibition of cell division [3, 4].

However, vinca alkaloids may induce apoptosis directly *via* upregulation of the p53 protein, a central regulator of apoptosis progression. In the breast cancer MCF-7 cell line, vincristine elevated p53 expression at the mRNA and protein level to the same extent as DNA damaging drugs [5]. Vincristine treatment was consistently associated with upregulation of the canonical p53-target genes, such as p21 and the growth arrest and DNA damage gene GADD45. Upregulated p53 is known to alter the balance of anti-apoptotic and pro-apoptotic proteins to favour apoptosis [6].

Unfortunately, applicability of vincristine in the treatment of cancer is limited by several side effects of this drug. The neurotoxicity of vincristine may represent an obstacle in its use in the treatment of neoplastic diseases and must be considered when the treatment protocol is adjusted for a particular patient [7].

The induction of the expression of several multidrug resistance (MDR) markers by vincristine, including the plasma membrane drug transporting P-glycoprotein (P-gp) [8], may directly affect the effectiveness of cancer chemotherapy and may be considered a real obstacle to future treatment.

P-glycoprotein, an ABCB1 member of the ABC transporter family, is most often described as the molecular cause of MDR [9, 10]. However, other multidrug resistance associated proteins (MRP), such as the members of the ABCC gene subfamily [11], and the breast cancer resistance protein (BCRP), an ABCG2 member of ABC transporter family [12], also confer drug resistance. While the efflux activities of P-gp and MRPs were reported to be involved in the depressed sensitivity of cells to vincristine [13], this chemotherapeutic is not extruded by BCRP [14]. Overexpression of P-gp as a consequence of the selective pressure of vincristine and several other anticancer agents could be proved in cell lines derived from human and animal malignancies. Examples for this possibility include mice leukemia L1210 cell variants that overexpress P-gp as an adaptation to vincristine or doxorubicin [15, 16].

Plasma membrane drug transporters such as P-gp were found to be upregulated in several cell lines treated by vincristine [13]. Vincristine-induced overexpressions of P-gp and other drug transporters are known to be predominantly transcriptionally regulated by the nuclear pregnane X receptor (PXR) [17]. However, the possible induction of the P-gp transporter by vincristine independent of PXR cannot be excluded [13]. The cytochrome P450 (CYP) family, particularly the CYP3A members, may be involved in the reduction of cell sensitivity to several dugs. The transcriptional control of the CYP3A is mediated by PXR, i.e., the same nuclear receptor involved in P-gp expression [18]. Cellular metabolism of vincristine seems to be related to high expression of CYP3A5 [19-21]. On the other hand, low expression of CYP3A5 is also associated with a higher risk of vincristine neurotoxicity [22].

Glutathione S-transferases (GST) represent a group of enzymes that are often involved in the protection of cells against toxic stress [23]. These enzymes catalyze the conjugation of several xenobiotics with reduced glutathione [24]. The actions of GSTs are often coordinated with MRPs that transport several conjugates of drugs and reduced glutathione [25]. The coordinated action of these two classes of multidrug resistance markers could also be deduced from the synergism of GST (isoenzyme M1) and MRP1 in the protection of melanoma cells against the cytotoxic effects of vincristine [26]. While P-gp is not able to transport glutathione conjugates, coordinated coexpression of P-gp and GST π was observed *in vitro* in human breast cancer MCF-7 cells selected for resistance by vincristine [27].

All the above facts are consistent with the acceleration of protective cell processes induced by vincristine occurring as a cellular response to vincristine toxic stress. These side effects of vincristine application represent an important

obstacle in altering success rates in cancer chemotherapy and must be considered in the design of treatment protocols. This article aims to describe the current state of knowledge about vincristine and its effects on MDR development.

Biochemical Characterization of P-Glycoprotein and Multidrug Resistance Associated Proteins

Plasma membrane P-gp was the first ABC transporter discovered in cancerous hamster ovary cells in 1976 [28]. This protein is encoded by the ABCB1 (mdr1) gene [29] and protects cells in a variety of tissues from the toxic stress caused by diverse endogenous and exogenous substances [9, 10, 30]. P-gp may be considered to be a transport membrane ATPase – an efflux pump with wide substrate specificity for several hydrophobic substances containing at least one tertiary amine (reviewed in [9, 10]).

P-gp is a polypeptide that consists of two similar halves. Each half is formed by a transmembrane domain consisting of six membrane-spans and an ATP binding site with an ABC motif consensus sequence [31]. This sequence is formed by two Walker regions, A and B, which are separated by 90 amino acids and are found in all known ABC proteins: (A – GXGKST and B – DEATSALD where X is an undefined amino acid). In ABC transporters, a C signature sequence (LSGG) is inserted between the A and B regions (separated from B by 20 amino acids), distinguishing ABC transporters from non-transporting ABC proteins (reviewed in [10, 31]). P-gp contains three putative glycosylation sites, corresponding to asparagines 91, 94 and 99 [32], and two phosphorylation sites for protein kinases A and C, which correspond to serines 669 and 681 [33].

The twelve transmembrane spans of P-gp were deduced to form a transmembrane pore with both ATP binding sites oriented towards the cytoplasm (reviewed in [9, 10, 31]). Drug binding sites should exhibit a complex architecture in which different parts of the site are responsible for binding to different drugs. Several lines of evidence indicate that the drug binding sites of P-gp are located at least partially in the membrane space. Transmembrane spans 1, 5, 6, 11 and 12 have been proposed to play different roles in the binding of various drugs to P-gp [34-40].

Multidrug resistance associated proteins (MRP1-7) are encoded by the ABCC/MRP subfamily of ABC genes [41-43]. They are involved in the plasma membrane transport of several mostly negatively charged substances, i.e., they are classified as anion transporters [44]. MRPs 1-3, 6 and 7 differ structurally from P-gp and other ABC transporters by the existence of an additional transmembrane domain formed by five transmembrane spans on the N-terminus [45]. After the N-terminal domain, this set of MRPs shows the typical structure of ABC transporters, i.e., two sequences form transmembrane domains consisting of six transmembrane spans and ATP-binding sites located in cytoplasm [46]. In contrast to P-gp, the N-terminus of MRPs is located in the extracellular space. Another structural feature typical for ABCC proteins is the structural diversity of two ATP binding sites which confers to the functional heterogeneity [47].

Although MRPs have been identified as organic anion transporters, and P-gp has been identified as a transporter of compounds containing at least one tertiary basic nitrogen atom [48], there is considerable overlap in their substrate spectrum [49]. This overlap can be seen by the fact that vincristine, doxorubicin and etoposide are all substrates of both P-gp and MRP1-3 [45].

Vincristine As Inductor of P-gp and MRPs – Insight to Drug Sensitivity of Cells

Expression of the ABCB1 gene takes place in response to diverse stress stimuli, including i. cytotoxic effects of diverse exogenous or endogenous substances; ii. hypoxia and reoxygenation insults; iii. deregulation of intracellular pH; iv. irradiation of cells with UV or γ beams (reviewed in [10]). In addition, many other anticancer drugs such as vinca alkaloids, doxorubicin, tamoxifen, docetaxel, cyclophosphamide, flutamide, ifosfamide, paclitaxel, and apicidin (an inhibitor of histone deacetylase) have been shown to promote the transcription of P-gp [15-17, 50]. Transcription of P-gp is mediated through increased activity of the mdr1 promoter region, which contains recognition sites for several transcription factors [51]. There are at least four nuclear receptors known to modulate the process of P-gp transcription: i. PXR (also known as a steroid and xenobiotic receptor) [52, 53]; ii. the constitutive androstane receptor (CAR) [17, 54-56]; iii) the vitamin D receptor (VDR) [57] and iv) the thyroid hormone receptor (TR) [57]. PXR was the receptor most

frequently described to control P-gp transcription [56]. This receptor is known as a promiscuous xenobiotic receptor [58], and a large spectrum of chemicals that includes vinca alkaloids [17] can induce its function. Moreover, vinca alkaloids can also activate another receptor active in P-gp transcriptional control – CAR [13]. Selection of a cell line with vincristine may yield a MDR cell variant strongly overexpressing P-gp. Expression and/or activity of this protein and the vincristine resistance of L1210 cells selected for resistance to vincristine (R) [16] were compared on Figure 2 with parental cells (S) and cells, in which expression of P-gp was fulfilled by stable transfection with the human P-gp gene (T) [59].

No considerable changes in the activity of GST [15, 60] and expression of MRPs [61] were observed in R cells when compared with S cells. However, coexpression of P-gp and MRPs as a consequence of activation of both PXR and CAR may occur. An example of this possibility is the activation of PXR and CAR in brain microvascular endothelial cells under treatment with antiepileptic drugs, leading to an elevation of P-gp, MRP1, and MRP2 expression [62]. Specific expression of multidrug resistance markers after selection with vincristine most likely depends on the type and function of the cells. This dependence could be documented for two cell lines derived from two patients with acute myeloblastic leukemia (AML) developed from myelodysplastic syndrome (MOLM-13 and SKM-1, supplied by DSMZ)[1]. Selection with vincristine in both cell lines yielded strong upregulation of mRNA encoding P-gp and downregulation of mRNA encoding MRP1 (Figure 3). However, in SKM-1 cells, but not in MOLM-13 cells, downregulation of mRNAs encoding BCRP and GST π1 was also observed.

PXR also regulates transcription of other players that are active in the alteration of cell resistance to diverse substances, i.e., cytochrome P450, particularly isoenzymes of the CYP3A subfamily. The substrates and inhibitors of P-gp overlap considerably with those of the CYP3A isoenzymes [63, 64]. The existence of common transcription factors involved in the induction of common substrates is a reason to suggest a functional interplay between P-gp and CYP3A isoenzymes [65] in the MDR phenotype.

However, induction of P-gp expression can also be observed when selection pressure from a substance that is not a P-gp substrate is used. This type of P-gp induction can take place if the substance is a ligand of PXR or of another nuclear receptor active in the control of P-gp transcription. Examples

[1] For details see http://old.dsmz.de/dsmz/main.php?menu_id=2).

for this behavior include cisplatin, which is known as a non-substrate of P-gp [66] but is a ligand of PXR [67, 68].

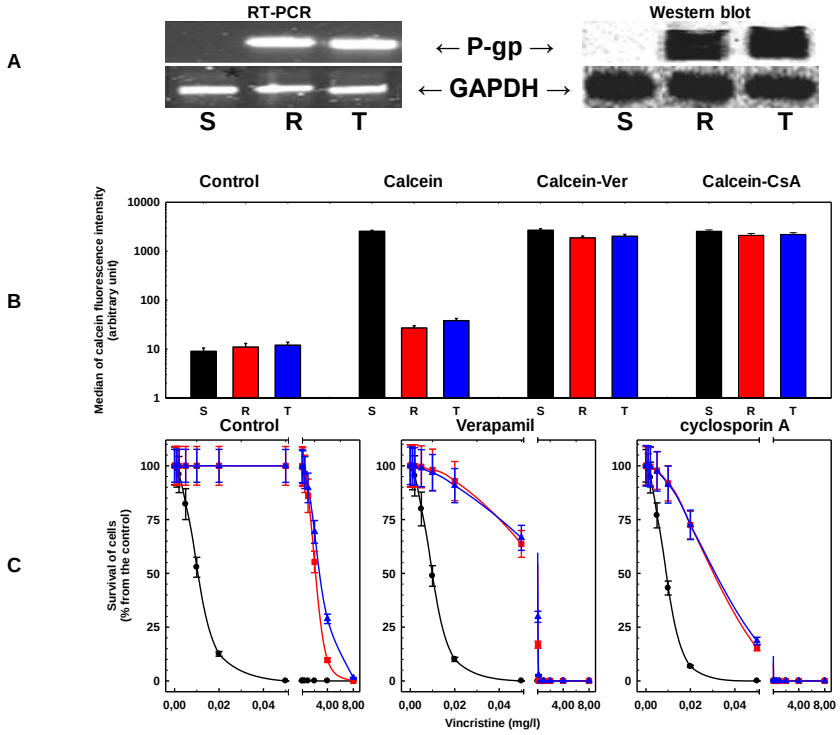

Figure 2. Characterization of P-gp mediated vincristine resistance in two P-gp positive L1210 cell variants obtained by selection with vincristine (R) and transfection with the human gene encoding P-gp (T) and compared with parental cells (S). **Panel A** – Expression of P-gp on mRNA and protein levels. Parental S cells did not contain measurable levels of P-gp mRNA and protein. Massive amounts of P-gp mRNA and protein are present in R and T cells. Panel B – Activity of P-gp measured by a calcein retention assay [144]. Calcein represents a fluorescent substrate of P-gp. While strong retention of calcein is present in S cells, the calcein efflux activity of P-gp diminishes this retention in R and T cells. Retention of calcein could be restored by the P-gp inhibitors verapamil (Ver, 10 μmol/l) and cyclosporine A (CsA, 0.8 μmol/l). Data represent the mean values±S.E.M from six independent measurements. Panel C – Cell death induced by vincristine measured by the MTT test [145]. While S cells exert strong sensitivity to vincristine, R and T cells are more than two orders less sensitive to this drug. Sensitivity to vincristine could be partially restored by Ver and CsA at the same concentrations which were described for calcein assay. Data represent the mean values±S.E.M from six independent measurements.

For this reason, cisplatin frequently induces P-gp expression [69-71]. Cells selected for resistance with cisplatin therefore often exert cross-resistance to several P-gp substrates. Similarly, resistance to the all-*trans* retinoic acid ATRA was linked to an improvement of P-gp expression [72], although this substance is not transportable by P-gp [73]. While nuclear receptors for retinoic acids (RAR) control the transcription of the CYP26 isoenzyme that metabolizes ATRA [74], this substance is also a PXR ligand and can induce overexpression of P-gp and CYP3A [75]. Interestingly, in P-gp positive L1210 cell variants, ATRA induces downregulation of P-gp levels and activity [31, 73].

The progression of drug-induced apoptosis in neoplastic cells is a key process in determining the effectiveness of many chemotherapeutics [76, 77]. P-gp eliminates drugs that are P-gp substrates from the intracellular space and consequently diminishes the apoptotic response.

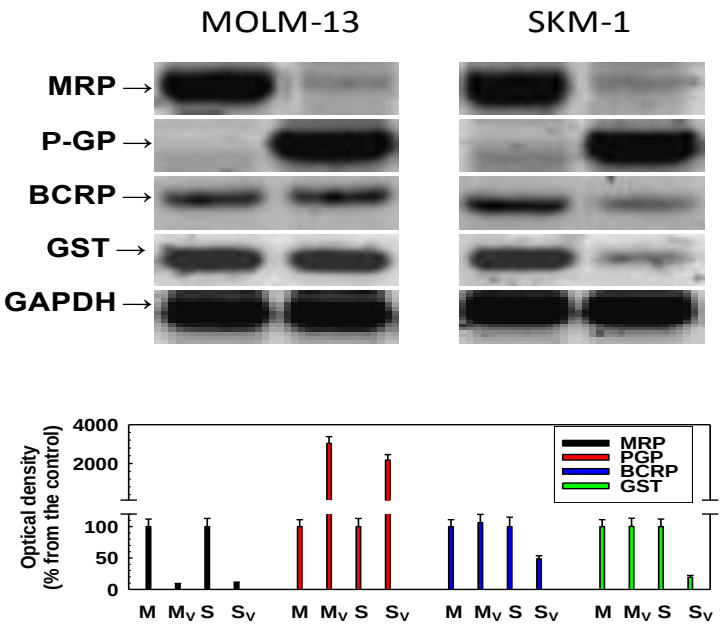

Figure 3. Expression of drug resistance markers in two AML cell models (MOLM-13 – M and SKM-1 – S) derived from patients in which AML developed from MDS. Cells were cultivated for three months in the absence (M, S) or presence of stepwise increasing concentrations of vincristine from 0.002 to 0, 5 mg/l (M_v, S_v). Upper panel – agarose electrophoresis detection of PCR products. Lower panel – quantification of band density. Data represent mean values±S.E.M from six independent measurements.

However, despite this widely accepted role of P-gp activity in apoptosis, this protein seems to play another drug-efflux-independent role involving modulation of the apoptotic pathway [78]. A dual protective effect of P-gp against toxic stress has recently assumed by Ruefli and Johnstone [79]. Both P-gp drug efflux activity and P-gp "anti-apoptotic" activity seems to be involved in the depression of toxin-induced apoptosis. The P-gp anti-apoptotic activity could imply i) P-gp as integral protein of plasma membrane interferes with the formation of the death-inducing signaling-complex in plasma membrane or ii) suppression of caspase activation *via* changes of either intracellular pH or changes in the activities of several chloride channels as a consequence of P-gp expression and activity [79].

The drug efflux-independent, anti-apoptotic effect of P-gp is unclear yet. However, strong evidence for its existence is illustrated by the fact that expression of a P-gp mutant defective in drug efflux activity in CEM lymphoma cells suppresses vincristine-induced apoptosis due to the reduction of mitochondrial cytochrome c release and caspase activation [80]. We have reported robust activation of caspase 3 and down-regulation of the anti-apoptotic Bcl-2 protein in mouse leukemia L1210 cells after treatment with cisplatin, which is a non-substrate for P-gp [61, 66]. Both, activation of caspase 3 and down-regulation of Bcl-2 were less prominent in the two P-gp-positive cell variants of L1210, R and T. Due to fact that Bcl-2 represents a substrate for caspase 3 [81], this protease could diminish the anti-apoptotic Bcl-2 protein content directly. The decrease of anti-apoptotic Bcl-2 cell content causes the prevalence of the pro-apoptotic Bax protein in the Bcl-2/Bax complex and therefore leads to cytochrome C release from mitochondria followed by additional caspase activation, DNA fragmentation and cell death [82].

Both P-gp and MRP are known to be involved in multidrug resistance. Cloning, functional characterization, and cellular localization of most MRP subfamily members have identified them as ATP-dependent efflux pumps localized in cellular plasma membranes with broad substrate specificity for the transport of endogenous and xenobiotic anionic substances (reviewed in [45]). Prototypic substrates include glutathione conjugates such as leukotriene C4 (for MRP1, MRP2, and MRP4), bilirubin glucuronosides (for MRP2 and MRP3), cAMP and cGMP (for MRP4, MRP5, and MRP8) and a wide range of therapeutic agents. [83].

Already in 1996, Loe et al. [84] obtained glutathione- and ATP-dependent vincristine transport in membrane vesicles isolated from MRP-transfected HeLa cells. The same group later showed that vincristine transport by MRP1

(ABCC1) requires reduced glutathione (GSH) for co-transport [85]. Moreover, the authors suggested that MRP1-mediated vincristine transport reaches a steady state when the GSH concentration reaches 5 mM. Vincristine was also demonstrated to be a suitable substrate for MRP2 (ABCC2); moreover, induction of MRP1 and MRP2 conferred resistance to vincristine [86]. Simultaneous activities of P-gp and MRP1 correlated with *in vitro* resistance to daunorubicin and *in vivo* resistance in adult AML patients [87]. A role for MRP1 in multidrug resistance of AML was proved and comprehensively discussed [88]. At the present time, several ABCC transporters have been reported in association with vincristine resistance, MRP1 and MRP2 as noted above, as well as MRP3 (ABCC3) and MRP7 (ABCC10) [45], and a non-ABC transporter - RLIP76 [89].

Cellular Modification of Vincristine and Vincristine-Induced Free Radical Formation – Insight into the Drug Sensitivity of Cells

In addition to several other anions, MRPs are known to transport the glutathionyl and cysteinyl conjugates of substances that represent products of the GST reaction, and this activity depends on the intracellular levels of reduced GSH and the activity of GST [90]. However, co-expression of MRP1 with any of the human GST isoenzymes failed to increase MRP1-associated resistance to drugs including doxorubicin, vincristine, etoposide, and mitoxantrone [25, 91]. The failure of co-expression of MRP1 with GST isoenzymes to increase MRP1-associated drug resistance might be evidence that vincristine is not conjugated to GSH but rather co-transported with GSH in MRP1-mediated drug resistance [90]. Vinca alkaloids are known to be suitable substrates for CYP3A isoenzymes that metabolize vinca alkaloids to less toxic compounds [92]. The monooxygenase reaction of this CYP subfamily may either deactivate or activate drugs; therefore, these enzymes are involved in modulation of the cell sensitivity to several substances. It was reported that the CYP3A subfamily is very often overexpressed in tumors under chemotherapeutic treatment *via* activation of xenobiotic the nuclear receptors PXR and CAR [93] and, furthermore, these enzymes are involved at least partially in depressed cell sensitivity to several drugs [94]. Vincristine is

metabolized by CYP3A4 and CYP3A5 to one dominant metabolite (M1) that represents a secondary amine with an opened ring (Figure 4) [19-21].

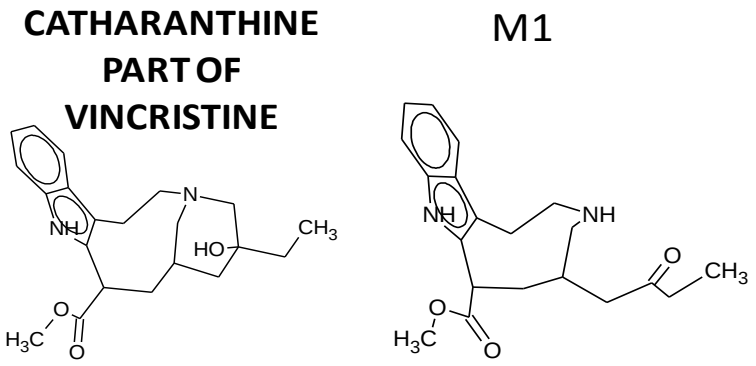

Figure 4. The dominant product of vincristine metabolized by CYP3A4 and CYP3A5 as described by Denninson and colleagues [19-21]. These enzymes modify only the catharanthine part of the vincristine molecule without any changes to the vindoline part. Only the catharanthine part is therefore documented. Structures were drawn by ACD/ChemSketch freeware software.

The presence of this metabolite was also confirmed clinically [22]. There are also other minor metabolites of vincristine as a product of CYP3A5 mediated reaction [21]. Although vincristine is not a proper substrate for glutathione S-transferase, a possible role of glutathione S-transferase in modulating the therapeutic effects of vincristine was suggested [26, 95, 96]. It could be speculated that the metabolites of VCR produced by CYP3A are more suitable substrates for the GST reaction than unmodified vincristine and, therefore, that its detoxification might be mediated by a contribution of glutathione S-transferase utilizing reduced glutathione.

Drugs such as vincristine and doxorubicin may induce cellular damage processes *via* elevation of the levels of free radicals [97, 98]. Both reduced and oxidized forms of glutathione together with glutathione peroxidase and glutathione reductase play a crucial role in the preservation of cellular redox status (i.e., oxidant antioxidant balance), and thus represent an important cellular defense system against free radical oxidation. This system could therefore contribute to a general chemoresistant phenotype [99]. Because the glucose-6-phosphate dehydrogenase (G6PDH) reaction is the major producer of cellular NADPH and NADPH plays an important role in the conversion of oxidized glutathione to its reduced form, the cellular content and activity of

G6PDH, as well as the availability of glucose, may alter cellular sensitivity to anticancer drugs such as vincristine [100].

It is known that vinca alkaloids may exhibit antitumor activity through the formation of reactive oxygen and nitrogen species (RONS) and through the RONS dependent activation of apoptotic cell death [101]. In this case, the modulation of oxidative stress by the antioxidant should antagonize the anticancer effect of vincristine [102]. However, effective antagonism by this process is not simple, and several processes antagonistic to drug cytotoxicity may follow in drug-induced oxidative stress. While oxidative stress in the short term induces inhibition of P-gp activity [103], chronic oxidative stress that induces GSH depletion might lead to up-regulation of P-gp expression [104]. On the other hand, both GSH and thiolated chitosan were found to depress P-gp ATPase activity [105]. Thus, to find an effective protocol for the use of antioxidants for the improvement of cancer chemotherapy, future detailed study will be necessary.

Effects of Vincristine on Cell Signalization – Insight to the Drug Sensitivity of Cells

Vincristine belongs to the group of microtubule targeting agents. Its anti-tumor actions are therefore associated with the disruption of the microtubule system and blockade of the cell cycle progression [106, 107]. Higher concentrations of vincristine (50 nM to 0.2 μM) were previously shown to induce G2/M arrest in cancer cells [108, 109].

Increasing evidence indicates that anticancer drugs activate signal transduction pathways and some of these pathways are associated with the progression of drug resistance in tumor cells [110]. The action of microtubule targeting agents such as vincristine has been shown to correlate with changes in activation of signal transduction pathways downstream of spindle damage or mitotic arrest. These pathways may play a pivotal role in the induction of apoptosis [111]. Mitogen-activated protein kinases (MAPKs) are known to play important role in the transduction of signals, and several reports have shown modulators of the MAPK pathways as effectors of P-gp drug transport activity in diverse multidrug-resistant cell models.

MAPKs are members of discrete signaling cascades that respond to different extracellular stimuli and mediate the regulation of important cellular

processes, including proliferation, differentiation and survival. They form the link between the outside world and the genome and are therefore important transcriptional regulators in adaptation to physical and chemical insults. Extracellular signal-regulated kinases (ERKs), p38-MAPK, and c-Jun N-terminal/stress activated protein kinases (JNKs) represent the dominant members of MAPK family. The ERK signaling pathway connects various membrane receptors to the nucleus and is activated in a variety of cell types by diverse extracellular stimuli. In HL60 cells, it was found that vincristine triggers the activation of both ERK and JNK-1 [111]. Pertussis toxin, a blocker of Go/Gi proteins, abrogated the vincristine-induced activation of both ERK and JNK pathways. A specific inhibitor of the ERK cascade, PD98059, markedly enhanced the induction of cell death by low concentrations of vincristine in a wide variety of tumor cells in which the ERK pathway is constitutively activated [112].

A role in the regulation of the MDR1 gene and chemoresistance in VCR resistant cells has been documented for another MAPK pathway, the cascade of p38-MAPK [113, 114]. The results showed that the p38-MAPK pathway was activated in both SGC7901/VCR and L1210/VCR cells, and the inhibition of this pathway decreased the levels or activity of P-gp.

The JNK pathway plays a pivotal role in the regulation of cell survival and cell death, depending on the specific cell type and context of activation. The activation of the JNK signaling pathway was shown to be associated with the promotion of apoptosis in several studies [115, 116], but it was also shown that transient activation of JNK can serve to both delay and reduce apoptosis in cardiac myocytes [117]. In this manner, JNK may be either a proliferative / anti-apoptotic (by activation with growth factors or cytokines) or pro-apoptotic (when activated by some cytotoxic chemicals such as vincristine) regulator of cellular functions. It has been documented that vincristine-induced microtubule damage results in JNK pathway activation and apoptosis induction in a EW36 B-cell line [115]. Moreover, the combination of a ERK pathway inhibitor with vincristine induced synergy in the activation of the JNK pathway, and this effect appeared to be responsible, at least in part, for the enhanced induction of cell death. In contrast, in our experiments inhibition of the ERK pathway in L1210/VCR cells potentiated vincristine cytotoxicity without an effect on the JNK pathway [118]. A recent study also indicates that JNK activation serves as a key mediator for vincristine-induced apoptosis in melanoma [119]. In addition, in human carcinoma cells (KB-3), treatment with VCR induced mRNA expression of MDR1 *via* mediation of the JNK pathway, which consequently yielded drug resistance [13, 120].

The functions of proteins active in switching between cell survival and apoptosis progression, i.e., proteins of the Bcl-2 family, are modulated through phosphorylation by diverse protein kinases including JNK [121-123]. Bcl-2 phosphorylation can occur in response to exposure of cells to specific classes of chemicals including disruptors of microtubule function, such as vincristine. Microtubule targeting drugs (taxols/vincristine) can also promote apoptosis by induction of the central apoptosis regulator p53 and its target protein p21 [122, 124]. On the other hand, several lines of evidence suggest that rearrangement of the cytoskeleton elevates focal adhesion kinase (FAK) activity with subsequent activation of PI3K/Akt and MAPK signaling pathways to perform an anti-apoptotic function [125].

An important role of the PI3K/Akt kinase pathway in vincristine-induced apoptosis is supported by findings that specific inhibition of the PI3K/Akt pathway markedly and selectively potentiated apoptosis induced by this microtubule destabilizer [126, 127]. Inhibition of mammalian target of rapamycin (mTOR) phosphorylation by Akt kinase was shown to enhance the apoptosis inducing effect of anti-microtubule agents [128].

Upregulation of P-gp expression has been documented to be dependent on activation of the PI3K/Akt signaling pathway [129, 130]. Moreover, a significant correlation between the phosphorylation of Akt kinase and P-gp expression has been described, and reversal of P-gp-mediated MDR due to inhibition of the PI3K/Akt signaling pathway has been documented [126]. Vincristine treatment increased phosphatidylinositol (3,4,5)-trisphosphate (PIP3) production by approximately 50% and simultaneously elevated activated Akt kinase content by specific phosphorylation [131].

However, it was recently reported that treatment of cells with microtubule-depolymerizing agents such as vincristine may increase survival potential by upregulating NF-κB expression and subsequent HIF-1alpha upregulation [132]. It was shown that depolymerization of microtubules activates NF-κB and induces NF-κB-dependent gene expression [133, 134]. Interestingly, P-gp was also found to be upregulated when NF-κB is activated [135, 136]. Modulation of the PI3K/Akt signaling cascade may activate the NF-κB pathway [131], and this activation is realized *via* phosphorylation of the inhibitory factor of NF-κB (IκB) by IκB kinases (IKKs). Akt kinase can also activate members of the mitogen-activated protein kinase (MAPK) family, indirectly affect IKK, and finally modulate NF-κB function [137, 138].

HIF-1 is involved in resistance to chemotherapeutic drugs, such as vincristine (VCR), in gastric cancer cells by induction of the anti-apoptotic

protein Bcl-2, inhibition of the pro-apoptotic protein Bax, or induction of multidrug resistance gene products P-gp and MRP [139]. These authors showed that various concentrations of vincristine may induce HIF-1 and VEGF expression and activity. Further study demonstrated that MGr1-Ag (a protein described to be upregulated in VCR-resistant cell lines) mediated vincristine-induced HIF-1 and VEGF expression by activation of FAK in SGC7901 cells [139]. Moreover, activation of MGr1-Ag and upregulation of HIF-1 could be functionally associated with the activation of the PI3K/Akt and MAPK signaling pathways by the action of vincristine.

In the mechanisms of cellular responses to vincristine action, other signaling molecules such as Aurora kinases or adenosine monophosphate activated protein kinase (AMPK) also play an important role. The Aurora family of serine/threonine kinases plays an important role in chromosome alignment, segregation, and cytokinesis during mitosis. It has been found that AZD1152, a selective Aurora B kinase inhibitor, potentiated the anti-proliferative activity of vincristine directed against PALL-2 and MOLM13 cells [140]. Vincristine alone increased the levels of the cleaved form of PARP, and the presence of AZD1152 also augmented the proportion of cleaved PARP. Aurora B plays a role in chromosome alignment, kinetochore-microtubule bioorientation, activation of the spindle assembly checkpoint, and cytokinesis in association with the phosphorylation of Ser10 in histone H3 [141].

In cultured melanoma cells, vincristine also induced activation of AMPK [142]. This activation of AMPK in B16, A-375, and WM-115 lines by vincristine was involved in the activation of p53 and inhibition of mTORC1, which may mediate the pro-apoptotic effects of AMPK. AMPK is a metabolic checkpoint downstream of the LKB1-tumor suppressor that integrates growth factor receptor signaling with cellular energy status. Recent studies indicate that AMPK activation induces phosphorylation of Ser15 in p53 in response to glucose starvation [143], which is required to initiate AMPK-dependent cell-cycle arrest and p53-dependent acceleration of cellular senescence.

Conclusion

While vincristine is a useful drug for the effective treatment of several neoplastic diseases, its action as an inductor of multidrug resistance should be considered when a proper chemotherapeutic protocol is prepared for a specific patient. This negative side effect could be diminished with several substances

known to modulate the expression and activity of MDR markers. It is generally accepted that combined therapeutic modalities are important to eradicate malignant disease. The application of modulators of glutathione status (such as specific inhibitors of glutathione-related enzymes or oxidative stressors), modulators of CYP activity and expression, and substances that alter the activity and expression of drug transporters in conjunction with anticancer drugs (including vincristine) may lead to the design of more effective treatment strategies. This represents an interesting subject for future focused research.

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