

VINCRIStINE AFFECTS THE REDOX REACTIVITY OF HEMOGLOBIN

VICTORIA CÎRJEU^a, CRISTINA PUȘCAȘ^a,
RADU SILAGHI-DUMITRESCU^{a*}

ABSTRACT. The anticancer drug vincristine, known to act by inhibiting tubulin function in human cells, is shown to affect autoxidation rates in hemoglobin – either alone or under conditions where nitrite acts as an initiator in a nitrosative stress cascade. This behavior mirrors those observed by us with four other antitubulin agents - irinotecan, docetaxel, paclitaxel and vinorelbine.

Keywords: *vincristine, hemoglobin, autoxidation, nitrite, oxidative stress, nitrosative stress, UV-vis*

INTRODUCTION

Anticancer agents have a wide variety of mechanisms by which they interfere with the growth of tumor cells. The largest group are agents that interact with DNA (by blocking DNA synthesis or by incorporation into DNA). Another important class of anticancer drugs is that of the antitubulin agents, which also affect cell division – but this time by interacting with protein scaffolds rather than with DNA [1–5]. Beyond these mechanisms, or often due precisely to them, chemotherapy can entail debilitating side effects such as bone marrow suppression, gastrointestinal damage, hair loss or nausea [1,2].

Hemoglobin (Hb) is found in large amounts in the blood and is among the first proteins in the human body to come into contact with anticancer drugs, so it is relevant to study the influence of cytotoxic compounds on it to understand the side effects of chemotherapy. Beyond its oxy/deoxy equilibrium in the ferrous state, Hb undergoes a range of redox reactions in blood as well as (or especially) in cases where it escapes the blood vessels

^a Babeș-Bolyai University, Faculty of Chemistry and Chemical Engineering, 11 Arany Janos str., RO-400028, Cluj-Napoca, Romania

* Corresponding author: rsilaghi@chem.ubbcluj.ro

in cases such as injury (e.g., subarachnoid hemorrhage). These redox reactions include autoxidation to ferric (met) Hb with liberation of superoxide and subsequently hydrogen peroxide, reaction with nitrosative stress agents such as nitrite, or reaction with oxidative stress agents such as peroxides. The chemical products of these reactions, as well as their biomedical relevance, have been explored to some length [6,7,16,17,8–15]. We have recently shown that cisplatin and related platinum complexes, as well as organic anticancer drugs and drug candidates, have the ability to affect the redox reactivity of hemoglobin – in terms of autoxidation rates, reactivity towards peroxides, and reactivity towards nitrite. These effects appear to entail Hb-drug complexes as well as direct redox reactions between the drug and hemoglobin and/or small-molecules stress agents [1,2,4,5,16,18–20].

Vincristine (IUPAC (3aR, 3a1R, 4R, 5S, 5aR, 10bR) -methyl 4-acetoxy-3a-ethyl-9 - ((5S, 7S, 9S) -5-ethyl-5-hydroxy-9- (methoxycarbonyl) -2, 4,5,6,7,8,9,10-octahydro-1H-3,7-methane [1] azacycloundecino [5,4-b] indol-9-yl) -6-formyl-5 -hydroxy-8-methoxy-3a, 3a1,4,5,5a, 6,11,12-octahydro-1H-indolizino [8,1-cd] carbazole-5-carboxylate), also known as leurocristine, is a colorless drug from the class of *Vinca* alkaloids used in chemotherapy to treat various types of cancer such as acute lymphocytic leukemia, acute myeloid leukemia, Hodgkin's disease, lung cancer, neuroblastoma and others. Vincristine is given intravenously, typically from preparations at ~ 1mM concentration and in amounts equating to 1 μM concentration in blood, and, like other *Vinca* alkaloids, binds to tubulin and blocks mitosis. X-ray analysis showed that the drug binds equally to α and β-tubulin and causes a curvature at the interface that leads to the spiraling of protofilaments, subsequently reacting with their unstable end and inhibiting protein polymerization, eventually resulting in cell death [21–26]. We have recently explored the reactivity of hemoglobin towards a set of four anti-tubulin agents - irinotecan, docetaxel, paclitaxel and vinorelbine – and found that they affect the Hb autoxidation rates (either alone or in assays where nitrite accelerates autoxidation via a cascade of nitrosative stress reactions) and, for members with more extended aromatic rings (and hence with measurable electronic absorption spectra in the visible domain), they can also serve as direct redox partners acting to reduce high-valent hemoglobin in the presence of peroxide [1].

Reported here is an exploration of Hb reactivity towards vincristine, employing UV-vis spectroscopy and showing similar behavior to that previously observed for other anti-tubulin agents – with two particularities: the effect on Hb autoxidation is very small, while the effect on nitrite-induced autoxidation is remarkably antioxidant.

RESULTS AND DISCUSSION

Figure 1 shows UV-vis spectra collected for oxy hemoglobin before and after incubation with 50 μM vincristine up to 4 hours. There appear to be no discernible differences in the Soret band at ~ 400 nm. However, closer inspection of the bands at 577 nm (specific to oxy Hb cf. Figure 1) and 630 nm (specific to autoxidized, met Hb, cf. Figure 1) does reveal an (albeit small) influence of vincristine in accelerating autoxidation. Table 1 further shows that the ratio of the oxy to met features (577 vs. 630 nm) in the Hb spectrum increases with vincristine concentration up to 75 μM .

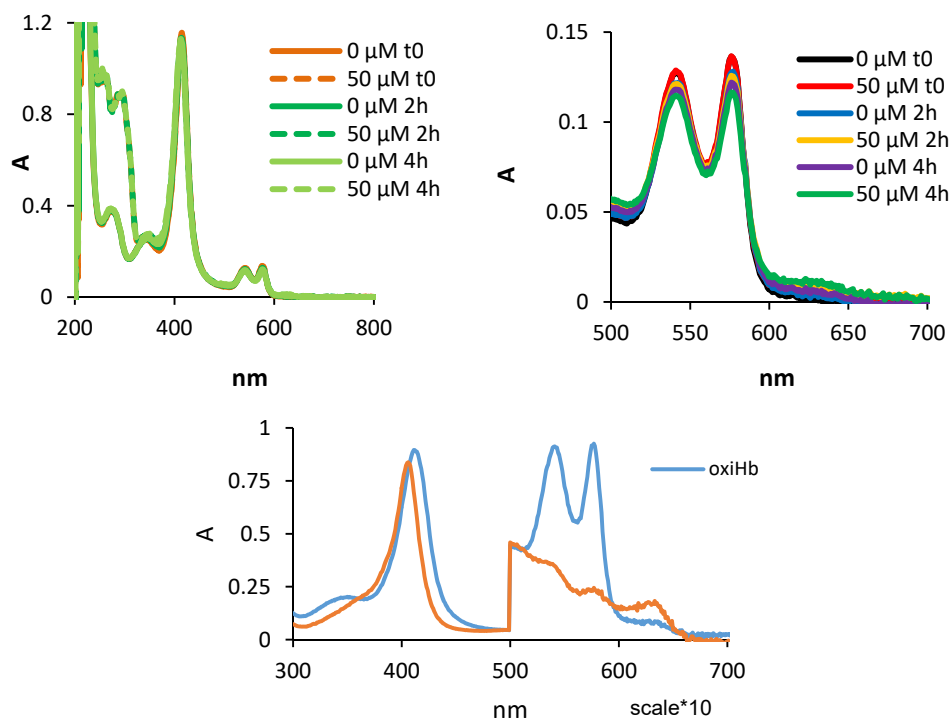


Figure 1. Top row: time dependence of oxy Hb UV-vis spectra with or without vincristine present. Lower row: reference spectra of oxy and met Hb.

Table 1. Met:oxygen ratio (calculated as the ratio of absorbance at 630 nm vs. absorbance at 577 nm) from UV-vis spectra collected after 4 hours at 37°C.

vincristine (μM)	0	10	25	50	75	100
met:oxygen ratio	0.05	0.07	0.07	0.09	0.12	0.08

The UV-vis spectra of red blood cells (Figure 2) are not affected by vincristine at concentrations of up to 50 μM immediately after mixing. However, after incubation at 37°C for 4 hours, the Soret band in the 50- μM sample is distinctly higher than that of the other two samples (0 and 10 μM vincristine, respectively). In erythrocytes the UV-vis spectrum is due almost exclusively to Hb; however, the intensity of the Soret band is additionally affected by the integrity of the cells: erythrocyte damage from oxidative stress upon Hb autoxidation appears to increase the intensity of the Soret band even when the 500-700 nm region reveals no change in the oxidation state of Hb [16]. We therefore propose that at 4 hours a 50 μM concentration of vincristine (but not a concentration of 10 μM) can induce optically-detectable changes in the state of the red blood cells. It is important to note that such cell lysis cannot be a direct result of Hb autoxidation; rather, autoxidation initially places oxidative stress (via the liberated superoxide) upon the cell. One may expect an initial phase where the cell structure remains intact but the antioxidant reserves (ascorbate, thiols, NADPH and others [27,28]) are consumed. Such a form of stress may then be expected to have manifested itself even in the 10 μM sample; this hypothesis would require subsequent tests on molecular markers of oxidative stress, other than Hb itself.

After incubation of erythrocytes overnight (as also shown in Figure 2), the Soret peak has now clearly shifted to 405 nm (specific to met Hb) from the initial 410 value in fresh (oxy) samples. Similarly to the 4-hour samples, there is a monotonous increase in absorbance from 800 to 200 nm, superimposed over the Hb absorption. This increase is due to diffuse/particulate matter in the sample, most likely protein precipitation; its degree does not appear to be increased proportionally with vincristine concentration.

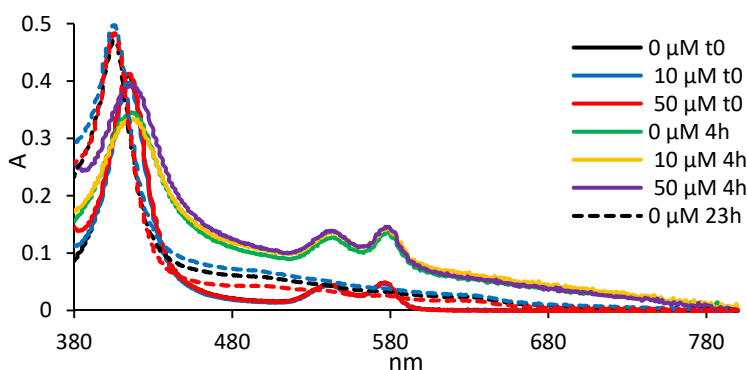


Figure 2. UV-vis spectra of erythrocytes re-suspended in saline phosphate buffer pH 7.4, with or without vincristine at two different concentrations, after incubation at 37°C.

Last but not least, Figure 3 shows the effect of vincristine on the nitrite-induced oxidation of Hb. In this assay, the initial step is reaction of nitrite with oxy Hb to form a caged ferric-peroxynitrate intermediate. However, clear changes in absorbance (e.g., at the oxy bands such as ~540 or ~580 nm) are not seen at these low nitrite concentrations until enough side-products (nitrogen oxides, superoxide, and others) accumulate. Hence, the key feature of the reaction time course is the lag time. Any added reactant (e.g., the vincristine) may be expected to affect the reaction either by delaying the change in absorbance (thus protecting the oxy form of Hb – and hence acting as antioxidant), or by shortening the lag time (thus acting as prooxidant). Figure 3 shows that already at a very low concentration, 30 μM , vincristine completely blocks the reaction – thus acting as a very efficient protecting agent against nitrosative stress on Hb.

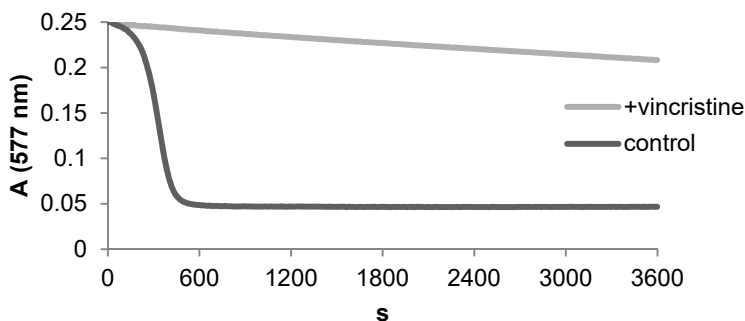


Figure 3. Influence of vincristine on the time evolution of oxy Hb absorbance in the presence of nitrite.

CONCLUSIONS

Vincristine has a very small (concentration-dependent) accelerating effect on Hb autooxidation *in vitro* in the concentration range 10-100 μM . At 50 μM , it also affects the stability of red blood cells. Vincristine also displays a powerful inhibitory effect on the (*in vitro*) nitrite-induced oxidation of Hb. The concentration ranges examined in the present study may be bio-medically relevant, since intravenous injections of vincristine in anticancer treatments are given with ~1000 μM solutions (although one must also note that after dilution in the total blood volume of a patient, even before the drug can exit to various organs in the body, the vincristine concentration would eventually not exceed 1 μM). Further examination at lower concentrations of vincristine

and on other types of cells would be warranted – though in those cases specific molecular markers would have to be measured, other than the very convenient Hb absorbance in UV-vis examined in the present study.

EXPERIMENTAL SECTION

Bovine hemoglobin was purified following the general protocol of Antonini and Brunori as previously described [29], and stored in saline (PBS), pH 7.4. In order to ensure a homogeneous (oxy) composition, the protein was first treated with sodium dithionite and passed through a desalination column. The hemoglobin concentration is given per heme. Vincristine was used as aqueous solutions prepared by mixing solid powder (drug) with saline (0.9% NaCl) without further purification. Electronic absorption were recorded with a Cary 50 instrument (Varian, Inc) in the range of 200-800 nm, in 1 mL quartz cuvettes.

Autoxidation of Hb (10 μM) in the presence of vincristine (at concentrations given in Figures) was monitored in PBS saline, pH 7.4, with the samples incubated at 37°C. Red blood cells were examined under similar conditions, using 30 μL of sample instead of 10 μM pure Hb.

Nitrite-induced autoxidation of was performed using oxyHb (240 μM), nitrite (334 μM) and vincristine (30 μM) in PBS at room temperature.

All results to be analyzed represent the average of at least 3 experiments.

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