Metallomics

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c9mt00174c

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

Antiproliferative activity of vanadium compounds: effects on the major malignant melanoma molecular pathways

Marina Pisano,[a] Claudia Arru,[a] Maria Serra,[a] Grazia Galleri,[b] Daniele Sanna,[a] Eugenio Garribba,[c] Giuseppe Palmieri[a] and Carla Rozzo*[a]

Malignant melanoma (MM) is the most fatal skin cancer, whose incidence has critically increased in the last decades. Recent molecular therapies are giving excellent results in the remission of melanoma but often they induce drug resistance in patients limiting their therapeutic efficacy. The search for new compounds able to overcome drug resistance is therefore essential. Vanadium has recently been cited for its anticancer properties against several tumors, but only a few data regard its effect against MM. In a previous work we demonstrated the anticancer activity of four different vanadium species towards MM cell lines. The inorganic anion vanadate(V) (VN) and the oxidovanadium(IV) complex $[VO(dhp)_2]$ (VS2), where dhp is 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinonate, showed IC_{50} values of 4.7 and 2.6 μM, respectively, against the A375 MM cell line, causing apoptosis and cell cycle arrest. Here we demonstrate the involvement of Reactive Oxygen Species (ROS) production in the pro-apoptotic effect of these two V species and evaluate the activation of different cell cycle regulators, to investigate the molecular mechanisms involved in their antitumor activity. We establish that VN and VS2 treatments reduce the phosphorylation of extracellular-signal regulated kinase (ERK) by about 80%, causing the deactivation of the mitogen activated protein kinase (MAPK) pathway in A375 cells. VN and VS2 also induce dephosphorylation of the retinoblastoma protein (Rb) (VN 100% and VS2 90%), together with a pronounced increase of cyclin-dependent kinase inhibitor 1 p21 ($p21^{Cip1}$) protein expression up to 1800%. Taken together, our results confirm the antitumor properties of vanadium against melanoma cells, highlighting its ability to induce apoptosis through generation of ROS and cell cycle arrest by counteracting MAPK pathway activation and strongly inducing $p21^{Cip1}$ expression and Rb hypo-phosphorylation.

Received 5th July 2019,
Accepted 21st August 2019

DOI: 10.1039/c9mt00174c

rsc.li/metallomics

Significance to metallomics

Vanadium compounds are promising anticancer agents. In this work, we demonstrate that two vanadium(IV,V) species exhibit antiproliferative activity towards the melanoma A375 cell line by arresting the cell cycle and triggering apoptosis through intracellular ROS production, ERK and Rb dephosphorylation and $p21^{Cip1}$ overexpression. These data can allow a better understanding of the mechanisms of action of potential vanadium anticancer drugs and provide information for the design of new active species.

Introduction

Melanoma is a heterogeneous malignant tumor that evolves from melanocytes and is one of the most aggressive tumors of skin and mucous membranes.[1] The epidemiology of melanoma is complex, and individual risk depends on sun exposure, host factors, and genetic factors, and on their interaction as well.[2] It is characterized by complex pathogenic mechanisms due to specific alterations in pathways controlling cell proliferation, differentiation and survival.[3] Melanoma incidence is still rising mainly because of screening campaign implementation, which has increased the number of reported melanomas. However, mortality due to melanoma is not decreasing.[4] About 85% of cutaneous melanomas concern the populations of North America, Europe and Oceania. In Italy, about 13 700 new cases

[a] *Istituto di Chimica Biomolecolare (ICB), Consiglio Nazionale delle Ricerche (CNR), Traversa La Crucca 3, 07100 Sassari, Italy. E-mail: carlamaria.rozzo@cnr.it*
[b] *Dipartimento di Scienze Mediche, Chirurgiche e Sperimentali, Università di Sassari, V.le San Pietro 8, 07100 Sassari, Italy*
[c] *Dipartimento di Chimica e Farmacia, Università di Sassari, Via Vienna 2, I-07100 Sassari, Italy*

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

of skin melanoma were expected in 2018, 7200 among men and 6500 among women (3% of all cancers in both sexes). In Italy, melanoma represents 9% of juvenile tumors in men and 7% in women; actually, it is the second and the third most common cancer under the age of 50, respectively.[5]

The genetics of melanoma comprises genes involved in pigmentation and naevi, as well as genes involved in the cell cycle and senescence, which have been identified *via* genome-wide association studies over the last 10 years and confirmed by next-generation sequencing (NGS)-based analyses of the entire genome or exome in melanoma patients.[6] A fundamental role in neoplastic development is attributable to alterations concerning various molecular pathways including that of the mitogen-activated protein kinases (MAPKs) involved in regulating cell growth. The uncontrolled activation of MAPKs is due to mutations within either the NRAS (Neuroblastoma RAt Sarcoma viral oncogene homolog; about 25% of cases) gene or the BRAF (v-Raf murine sarcoma viral oncogene homolog B; about 50% of cases) gene, which can present a substitution of valine in position 600 (V600).[3] The last codon change represents the most common variation in patients carrying a mutated BRAF (roughly, 90% of BRAF mutations).[3] These gene mutations determine the constitutive activation of the B-Raf protein kinase (cytoplasmic protein), which induces the activation of the mitogen-activated protein kinase (MEK), which in turn activates the extracellular-signal regulated kinase (ERK), the final effector of the cascade, inducing the transcription of target genes that trigger the cell entering the cell cycle.[7]

The determination of mutated oncogenes as specific targets has allowed the development of so-called targeted therapies or personalized therapies as they can be adopted only in the case of patients carrying specific alterations. With regard to BRAF mutations, mutated BRAF inhibitors were synthesized, including vemurafenib, dabrafenib and encorafenib, which strongly inhibited MEK phosphorylation, and deactivated the MAPK cascade, blocking cell growth in melanomas.[8] Concerning NRAS mutations, no specific inhibitor is available; treatment with MEK inhibitors is effective in melanomas with oncogenic activation of the NRAS-BRAF cascade.[3,9,10] The combination of BRAF and MEK inhibitors, alone or associated with immunotherapy, has been demonstrated to produce tumor responses in the majority of metastatic melanoma patients carrying BRAF V600 mutations or, to a much lesser extent, NRAS mutations.[11] Indeed, most melanoma cases present activating mutations within the MAPK pathway but, unfortunately, the produced responses are largely partial and not durable. Actually, activation of alternative pathways controlling cell proliferation generally induces resistance to these treatments in a few months.[12] Due to the complexity of the molecular mechanisms involved in the pathogenesis of melanoma and the appearance of resistance to target drugs, it is appropriate to consider the development of new drugs with more broad-spectrum action to be associated with such inhibitors with specific action. They could be considered in order to undertake effective combined therapies, or otherwise to face the onset of resistance.[13] In this context, new drugs characterized by the presence of metals, in particular vanadium, can be taken into consideration and represent the subject of this work.

The development and use of metal-based drugs in cancer treatment is mainly linked to the antitumor effects demonstrated for platinum-based compounds, among which cisplatin is the progenitor and is currently used as a conventional chemotherapeutic agent in antitumor clinical therapy.[14] Metal-based drugs have very similar functioning, as they share some molecular mechanisms and in particular the ability to bind to DNA in a covalent manner, triggering the process of cell death. In addition to platinum other metals have shown excellent antitumor properties such as compounds based on gold, arsenic, antimony, bismuth, vanadium, iron, rhodium, titanium and gallium.[15–18] These metals and their compounds may have a divalent action: some may induce tumor development while others may counteract cancer and some of these compounds may act in both ways.[19]

Among the metals listed above, vanadium, a transition metal of the fifth group, and its compounds, have catalyzed researchers' attention for their possible use in the medical field.[20–23] V is an element present in organisms in ultratraces (the amount that is taken in through the diet is on average less than 1 mg per day); it is present in tissues with a concentration of some μg per kg and is thought to be essential for normal metabolism, growth and development of mammals. Some vanadium compounds have been used for their insulin-mimetic action and for the reduction of hyperlipidemia and hypertension.[24] Vanadium-based compounds have also been proposed for the treatment of neuronal and cardiac disorders, in the treatment of bacterial, viral and parasitic infections and in the treatment of malignant tumors.[25,26]

The first studies on the potential application of vanadium salts in the treatment of cancer date back to 1965[27] and in 1986 the first work appeared describing the action of an organometallic vanadium compound, vanadocene dichloride, $[Cp_2V^{IV}Cl_2]$, in the treatment of Ehrlich tumors.[28] Several subsequent studies have shown the potentiality of vanadium as an anticancer drug as reviewed by Crans and colleagues.[29] Gomez-Ruiz *et al.*[30] reported that vanadocene dichloride and some derivatives have a high cytotoxic activity like other metallocenes of formula $[Cp_2MCl_2]$, where M = Ti, Zr, or Mo. Initially it was proposed that these complexes bind to the nitrogenous bases and/or DNA phosphate, a type of interaction that resembles that of cisplatin.[31] Another possible mechanism of action against tumor cells could be inhibition by the vanadocene of topoisomerase II, which should occur when the complex $[Cp_2V^{IV}Cl_2]$ interacts with the enzyme groups that are present on its surface, causing a reduction of the enzymatic activity.[32] Among the derivatives of oxidovanadium(IV), Metvan (bis(4,7-dimethyl-1,10-phenanthroline)-sulfatooxydovanadium(IV)) has been identified as one of the most promising antitumor multitarget compounds of vanadium.[33] It has been described to be able to induce apoptosis in leukemia, multiple myeloma, and solid tumors such as breast, prostate, testis, and glioblastoma, and it is highly effective against cisplatin-resistant ovarian and testis tumor cell lines.[34,35] Recently, growing attention was focused on the antitumor activity of vanadium complexes formed by flavonoids,

 This journal is © The Royal Society of Chemistry 2019

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

which are very effective against osteosarcoma cell lines.[36–38] It was proposed that the generation of reactive oxygen species (ROS) causes a series of cellular effects, such as DNA cleavage and protein tyrosine phosphatase (PTPases) inhibition;[36] moreover, the activation of ERK phosphorylation promoted by the $V^{IV}O$-quercetin species seems to be involved in one of the possible mechanisms explaining the pharmacological effects of this compound.[39] Several reviews have been published over the last years and interested readers are referred to such studies.[20,29,31,40–42]

It has been reported in the literature that vanadium-based compounds exert antiproliferative and cytotoxic activity on human pancreatic tumor cells, inducing cell cycle blockage in the G2/M phase and producing an increase of ROS.[43] At high concentrations, vanadium salts were able to inhibit the formation of colonies of human tumor cells by blocking the cell cycle in the G2/M phase. This effect seemed to be due to the inhibitory action that vanadium plays on PTPase, whose task is to dephosphorylate the cyclin dependent kinase (CDK)–cyclin B complex, allowing the cell cycle to progress.[44] It has also been shown that the reduction of the proliferation induced by some vanadium compounds is associated with the inhibition of the cyclins D1 and E and the E2 transcription factor (E2F) and the induction of tumor suppressors cyclin-dependent kinase inhibitor 1 p21 ($p21^{Cip1}$) and kinase inhibitor protein 1 p27 ($p27^{Kip1}$).[45] Vanadate induces activation of the mammalian target of the rapamycin (mTOR)/S6 ribosomal protein (S6R) pathway downstream of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) signal, as well as DNA fragmentation, loss of membrane potential in mitochondria, ROS production, and caspase-3 activation.[46] As mentioned before, vanadium exerts its antiproliferative activity also through the induction of ROS formation.[20] ROS are crucial agents in the cell fate. Their intracellular accumulation in normal cells leads to the oxidation of various cellular components such as nucleic acids, proteins and lipids. These oxidative reactions cause extensive damage and in cases of irreparable damage they promote apoptosis.[42]

On the basis of these observations we synthesized some compounds based on vanadium and tested them for their cytotoxic activity against malignant melanoma (MM) cells, as previously reported.[47] Two of these compounds have shown promising antitumor activity, being less effective on normal control cells: an inorganic vanadium(V) salt, sodium vanadate (VN), which at physiological pH exists mainly in the anionic form $H_2VO_4^-$, and a neutral oxidovanadium(IV) complex with 1,2-dimethyl-3-hydroxy-4(1*H*)-pyridinone (abbreviated to dhp), $[VO(dhp)_2]$ (VS2) (Fig. 1). In our previous study, we demonstrated the antitumor activity of such compounds on MM cells and, in particular, their ability to induce apoptosis and cause cell cycle arrest at different phases (VN blockade in phase G2 and VS2 in phase G1).[47] Starting from these previous results, in this study we decided to better characterize the molecular mechanisms underlying the antitumor properties of vanadium compounds observed with melanoma cells. For this purpose we chose to utilize A375 melanoma cells,[48] which have been previously described as a low metastatic cell line,[49,50] resembling the most frequent disease condition among melanoma patients (studies of a large series of them indicated that the average incidence of disease relapses is about 40% and the most frequent first sites of recurrence during follow-up are the regional lymph nodes, expressing a lower metastatic potential as compared to that of the distant metastases).[51] Moreover, A375 is considered as a reference cell line for MM in *in vitro* studies (with more than 1000 publications of studies using A375 melanoma cells reported in PubMed), and was shown to be the most sensitive to our V compounds, useful as a model to better dissect the molecular mechanisms of action of VN and VS2. The aim of this work is, in fact, to understand how vanadium compounds can cause arrest of proliferation and trigger programmed cell death in melanoma cells. Therefore, here we evaluated the ability of these compounds to generate ROS in A375 cells and whether this was involved in the induction of apoptosis that we had already observed in MM cells. Moreover, we investigated more in-depth the mechanisms involved in cell cycle arrest by evaluating the expression and/or activation of some molecular markers of cell cycle progression, which are components of pathways involved in the onset and progression of melanoma, in A375 cells treated with VN and VS2. In particular, we analyzed: the ERK kinase (to assess the MAPK pathway activation status), the retinoblastoma protein (Rb) (which represents an important control point for the transition from the G1 phase to the S phase), the cyclin/CDK complex inhibitor $p21^{Cip1}$ (to verify its possible involvement in cell cycle blockade), and the cell division cycle 25C phosphatase (Cdc25C, whose activation is necessary for the transition from phase G2 to phase M).

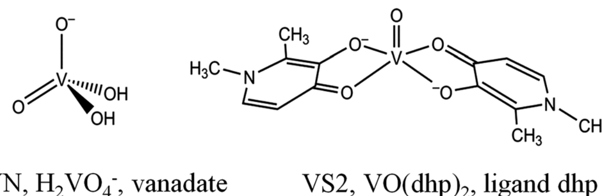


Fig. 1 Chemical structure of the vanadium compounds studied in this work (adapted from ref. 47).

Experimental

Cell culture

The A375 MM cell line was purchased from the ATCC (American Type Culture Collection, ATCC® #CRL-1619™). It was previously genetically characterized and presents the BRAF V600E mutation (the substitution of a valine residue (V) with a glutamate (E) in position 600) as well as two point mutations in the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, E61* and E69*, both due to one-base pair substitution ending in substitutions of glutamate residues (E) at position 61 and 69 into two stop codons, which impair the expression of $p16^{INK4A}$ and $p14^{INK4B}$ (inhibitors of CDK4) tumor suppressor proteins.[52] Cells were grown using RPMI culture medium with stable glutamine, supplemented with 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

(1 U mL^{-1}) (complete medium), at 37 °C in a humidified atmosphere with 5% CO_2.

Vanadium compounds and chemicals

Two different vanadium compounds were used: $[VO(dhp)_2]$ (VS2) and inorganic vanadate(V) (VN) (Fig. 1). $[VO(dhp)_2]$ was prepared according to the established procedure.[53,54] The VS2 stock solutions were prepared always immediately before use by dissolving, in complete RPMI medium, weighed amounts in order to have a concentration of 5 mM. The VN stock solutions were also prepared immediately before use by dissolving NaV^VO_3 in PBS at pH 7.4 to reach a concentration of 10 mM. At this concentration, polynuclear species tend to form, including decavanadate, which shows a yellow colour; this species can be easily decomposed by heating.[55]

N-Acetyl-L-cysteine (NAC, Sigma-Aldrich) stock solution (500 mM pH 7.4) was prepared by solubilizing a weighed amount in a 50 mM phosphate, 150 mM NaCl buffer solution, titrated to pH 7.4 with the addition of NaOH.

The stability and speciation of VN and VS2 in the RPMI culture medium were previously discussed by some of us.[47]

Antibodies

Primary antibodies anti-ERK1/2 (#13-6200), anti-phosphoERK1/2 (#700012) and anti-phosphoCdc25C (#MA5-15146) were from Thermo Fisher Scientific, anti-p21^{Cip1} (#2947) and anti-phosphoRb (#8180) were from Cell Signaling, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#SC-47724) was from Santa Cruz Biotechnology. Secondary antibodies, horse-radish peroxidase (HRP) conjugates, were anti-mouse (#AP124P) from Millipore and anti-rabbit (#A27036) from Sigma-Aldrich.

ROS assay

Reactive Oxygen Species (ROS) were evaluated using the CellROX® green flow cytometry assay kit (#C10492, Thermo Fisher Scientific), which enables the cytometric detection of ROS in live cells. A375 cells were seeded in 6-well plates at the optimal density depending on the experimental times (for 4 and 24 hours at 3×10^5 cells per well, while for 48 hours at 2.5×10^5 cells per well). After 24 hours the medium was removed and replaced with fresh medium containing the two vanadium compounds (VN and VS2) at two different concentrations (10 and 20 μM), with and without NAC (5 mM). A positive control was prepared by treating cells with *tert*-butyl hydroperoxide (TBHP), a known ROS inducer, while the negative control consisted of cells treated with the antioxidant agent NAC, as suggested by the manufacturer instructions. After treatment, cells were harvested by adding 400 μL of 0.25% trypsin and collected through centrifugation (10 min at $200 \times g$, where g is the relative centrifugal force, at room temperature (RT)). Pellets were resuspended in fresh medium, and incubated with 1 μL of Cell ROX fluorescent dye in the dark for 30–60 min. Samples were then analyzed using a FACS Canto II (BD Biosciences, San Jose, CA, USA) flow cytometer and the results evaluated using the ModFit LT 3.0 software (Verity Software House).

Apoptosis assay

Apoptosis assays were performed using the ''Tali™ Apoptosis Kit – Annexin V Alexa Fluor® 488 and Propidium Iodide'' (Life Technologies). This assay is based on the detection of the green fluorescent Annexin V, labeled with Alexa Fluor, bound to the phosphatidylserine exposed on the cell membrane of apoptotic cells. Propidium iodide (PI), instead, binds to the exposed DNA of dead cells and is used to discriminate between necrotic (red) and apoptotic cells (green). Cells showing double fluorescence green/red are considered as in the late stage of apoptosis.

Apoptosis assays were performed on A375 cells treated with 10 and 20 μM of VN and VS2 for 24 and 48 hours, with and without 5 mM NAC. Cells were seeded on 6 well plates at the optimal density depending on the experimental times (3×10^5 cells per well for 24 hours; 2.5×10^5 cells per well for 48 hours). In order to inhibit ROS production, 30 min before the vanadium compound treatment, control samples were incubated with 5 mM NAC. Sample analyses were performed following the manufacturer instructions and as previously reported,[47] except for the final sample analysis carried out using a FACS Canto II (BD Biosciences, San Jose, CA, USA) flow cytometer. Results were evaluated using the ModFit LT 3.0 software (Verity Software House).

Cell cycle assay

Cell cycle analyses were performed using the ''Tali® Cell Cycle Kit'' (Life Technologies), which provides a propidium iodide solution, a red fluorescent dye that binds DNA after cell permeabilization, allowing the quantification of the cellular DNA content during cell cycle progression. The experimental procedure was performed following the manufacturer instructions and as previously reported[47,56] with some differences briefly described: A375 cells were seeded on 6 well plates at 2.5×10^5 cells per well for 24 hours. Immediately after, the medium was replaced by fresh medium containing VN or VS2 compounds at 10 and 20 μM for 24 hour treatment. Then, the cells were harvested, washed with PBS and fixed with 1 mL of 70% ice-cold ethanol. After incubation at −20 °C overnight, the samples were centrifuged, resuspended in 200 μL of Tali® Cell Cycle Solution and kept for 30 min in the dark at RT. Red fluorescence was measured by means of a FACS Canto II (BD Biosciences, San Jose, CA, USA) flow cytometer and the results were evaluated using the ModFit LT 3.0 software (Verity Software House).

Protein expression analysis

Cell lysis and protein extraction. Cells were seeded in T25 tissue culture flasks in complete medium and grown to semiconfluence, and then were treated for 3, 6, 16 and 24 hours with medium containing or not 10 and 20 μM VN or VS2. The cell density was 3×10^6 cells per flask for 3 and 6 hour treatments while 2.5×10^6 cells per flask for 16 and 24 hour treatments. Immediately after, cells were harvested using a cell-scraper and lysed with cold Pierce™ RIPA lysis buffer (#89900, Thermo Scientific) containing protease and phosphatase inhibitor

 This journal is © The Royal Society of Chemistry 2019

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

cocktail 1× (#78441, Thermo Scientific) and pepstatin A (1 mg mL^{-1}) (#516481, Sigma-Aldrich). Following incubation of 15 min on ice, cell lysates were collected and centrifuged at 18 000 × *g* at 4 °C for 15–20 min. The supernatants were recovered, aliquoted and stored at −80 °C. The protein concentration was determined by the QuantiPro BCA Assay Kit (Sigma-Aldrich) using a standard reference curve with different concentrations of Bovine Serum Albumin (BSA) (0–100 μg mL^{-1}).

SDS PAGE and Western blotting. Protein lysates (30 μg proteins per lane) were resolved using pre-cast 12% or 4–12% gradient polyacrylamide gel (NuPAGE Bis-Tris Gel, Invitrogen), depending on the size of the proteins to be detected, and transferred to iBlot® Gel Transfer Stacks Nitrocellulose by an iBlot™ Dry Blotting System, Mini (all from Invitrogen). Membranes were then incubated with primary antibodies overnight at 4 °C, except for anti GAPDH, which was incubated at RT for 1 hour. Secondary antibodies, HRP-conjugated anti-mouse or HRP-conjugated anti-rabbit, were incubated for 1 hour at RT. Immune complexes were visualized with the use of an enhanced chemiluminescence system (Pierce® ECL Plus Blotting Substrate kit, Thermo Scientific) using an UVITEC mini HD6 imaging system, which quantified protein levels. Data were finally analyzed with the UVITEC UVI-D1 software.

Results and discussion

In a recent paper we evaluated the antiproliferative effect of four vanadium compounds (VN, VS2, VS3 and VS4) comparing the activity of the free vanadate (VN) with that of vanadium bound to different ligands (VS2, VS3, and VS4); VS3 and VS4 were two derivatives of VS2, [$V^{IV}O(mpp)_2$] and [$V^{IV}O(ppp)_2$], with mpp 1-methyl-3-hydroxy-4(1*H*)-pyridinonate and ppp 1-phenyl-2-methyl-3-hydroxy-4(1*H*)-pyridinonate.[47] Among them, the compounds VN and VS2 (Fig. 1) proved to be more efficient against the A375 melanoma cell line, showing IC_{50} values of 4.7 and 2.6 μM, respectively. These two compounds showed instead much higher IC_{50} values for normal fibroblasts (BJ) used as control cells (10.5 μM and 14.5 μM for VN and VS2, respectively). We have also demonstrated that VN and VS2 were able to induce apoptosis in a dose- and time-dependent manner and to cause cell cycle arrest at different phases (VN caused a blockage in the G2 phase, while VS2 in G1). Based on these data, here we want to investigate the molecular mechanisms of such antiproliferative activity of these two compounds using the A375 melanoma cell line, the most sensitive among those tested, as an *in vitro* experimental model of MM.

Induction of ROS production by VN and VS2

Oxidative stress caused by the production of intracellular ROS is one of the most reported effects exerted by vanadium, intimately linked with the proapoptotic activity of several vanadium compounds in tumoral cells.[20,42,43,57–59]

Therefore, we wanted to assess whether ROS production could play a role in the induction of apoptosis caused in A375 melanoma cells by VN and VS2. In order to verify ROS production due to VN and VS2 treatment, A375 cells were grown in the presence of both vanadium compounds at 10 and 20 μM for three experimental times (4, 24 and 48 hours). A sample of cells for each experimental point was also pretreated with NAC, a general ROS scavenger.[60] The levels of ROS in each sample were quantified through cytofluorimetric analysis as described in the "Experimental" section. The obtained results are shown in Fig. 2(A–C): a general increase in the amount of ROS in VN- and VS2-treated cells was observed for all three experimental times compared to the untreated cells, reaching about 80% of ROS positive cells after 48 hours. This finding demonstrated that both compounds caused induction of ROS production. When the cells were pretreated with

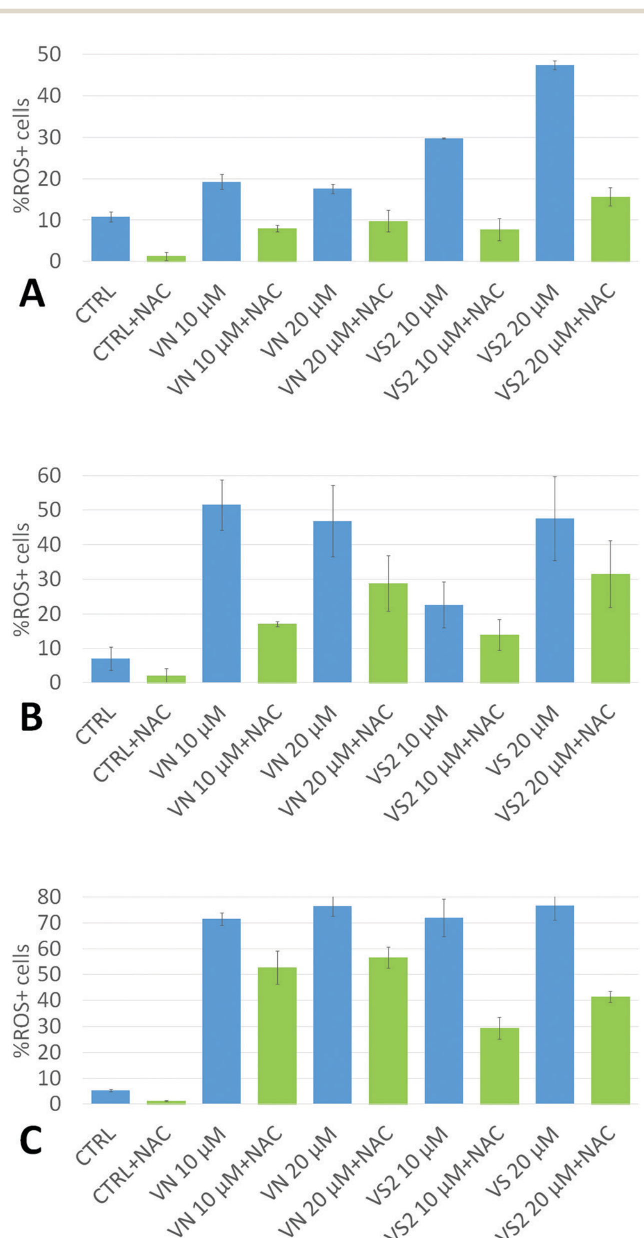


Fig. 2 Evaluation of ROS production. The graphs show the percentage of ROS positive A375 cells after 4 hour (A), 24 hour (B) and 48 hour (C) treatments with 10 and 20 μM of either VN or VS2 compounds, with or without 5 mM NAC, as described in the "Experimental" section.

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

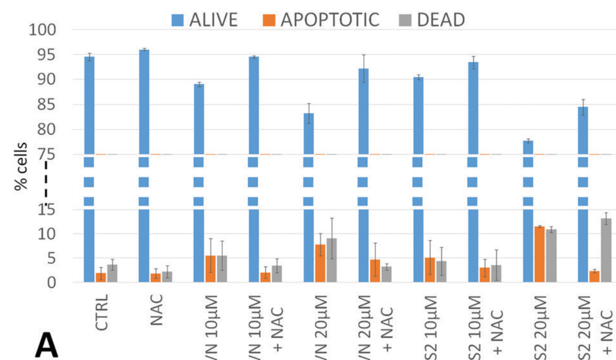
the antioxidant agent NAC, the percentage of ROS decreased significantly, due to NAC counteracting the oxidative effect of the vanadium compounds. The increase of ROS was time-dependent, and it was evident after only 4 hours of treatment, especially for the VS2 compound (up to 48% of ROS positive cells). It has to be noticed that while after the 4 hour treatments VS2 (at both concentrations 10 and 20 μM) showed greater ROS production compared to VN (19% of ROS positive cells, Fig. 2A), in the 24 hour treatments the situation was reversed, in fact VN caused slightly higher ROS production compared to VS2 (53% VN *vs.* 45% VS2, Fig. 2B). After 48 hours of treatment, the two compounds showed a very similar trend (about 75–80% for both compounds, Fig. 2C).

VS2 also showed a dose-dependent trend of this increase, more evident for the shorter treatment times (4 and 24 hours, Fig. 2A and B), while at 48 hours almost a plateau was reached and the quantities of ROS detected at 10 and 20 μM were similar (70–75%, Fig. 2C). For vanadate(V) (VN), instead, a dose-dependence effect has not been observed, in particular at 4 and 24 hours, where the percentage of ROS detected at VN 10 μM was very similar to, even slightly higher than, that observed with VN 20 μM (Fig. 2A and B). With regard to the different behavior of the two compounds it should be noted that V(IV) can be easily oxidized to V(V) and *vice versa* V(V) can be reduced to V(IV). In the cellular environment the most stable oxidation state should be the +IV one, which is that of VS2, while VN, with vanadium in oxidation state +V, after crossing the cell membrane should be reduced to V(IV) by the cell reducing agents (*e.g.* glutathione).[61] The non-complexed V(IV) and its compounds are able to give Fenton-like reactions[62] leading to the formation of ROS according to the reaction: $V^{IV}O^{2+} + H_2O_2 \rightarrow V^{V}O_2^{+} + H^{+} + {}^{\bullet}OH$. Therefore, the whole process can be described as a complex mechanism involving the redox interchange of the +V and +IV oxidation states and the capability of V^{IV} species to generate ROS through the Fenton-like reaction. It must be also considered that at physiological conditions the formation of polyoxovanadates is possible and these can induce an increase of the ROS level.[63,64]

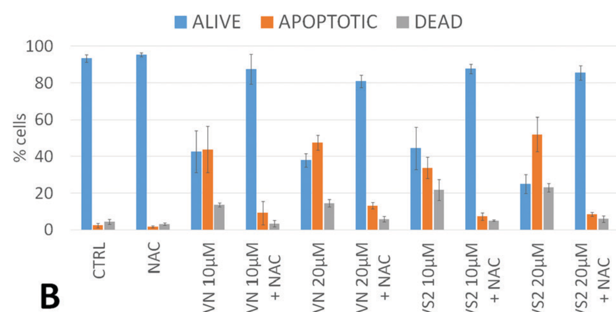
However, the results obtained confirm, decisively, the ability of vanadium and its compounds to determine a state of oxidative stress on various tumor cell lines including those of melanoma as reported in the literature.[65]

Apoptosis is induced by ROS production

Having ascertained the production of ROS in A375 cells following treatment with the two compounds VN and VS2, we verified if this was one of the causes of the apoptotic process observed previously.[47] Also in this case the cells were treated with the two compounds (VN and VS2) at concentrations of 10 and 20 μM, for 24 and 48 hours, with or without the presence of 5 mM NAC, and were submitted to the Annexin V assay. The results, shown in Fig. 3, confirmed the induction of apoptosis with a dose-dependent trend, highlighted a little after 24 hours (8% and 11% of apoptotic cells with 20 μM VN and VS2, respectively, Fig. 3A) but very clear at 48 hours for both the compounds (47% and 52% of apoptotic cells with 20 μM VN and VS2, respectively, Fig. 3B). In the samples pretreated with 5 mM NAC, we observed a reduction of apoptotic cells from 48% to 13% in VN treated cells, and from 52% to 9% in VS2 treated cells, thus demonstrating that the scavenging of produced ROS counteracted the induction of apoptosis triggered by the vanadium compounds, thus confirming the effect of ROS on apoptosis. Our data confirm the effect of ROS on apoptosis previously reported by Strianese and colleagues for the same A375 melanoma cell line treated with a pyridoxal-based vanadium(IV) complex.[65]



24 h	CTRL	NAC	VN 10μM	VN 10μM + NAC	VN 20μM	VN 20μM + NAC	VS2 10μM	VS2 10μM + NAC	VS2 20μM	VS2 20μM + NAC
ALIVE	95%	96%	89%	95%	83%	92%	90%	93%	78%	85%
APOPTOTIC	2%	2%	6%	2%	8%	5%	5%	3%	11%	2%
DEAD	4%	2%	6%	3%	9%	3%	4%	4%	11%	13%



48 h	CTRL	NAC	VN 10μM	VN 10μM + NAC	VN 20μM	VN 20μM + NAC	VS2 10μM	VS2 10μM + NAC	VS2 20μM	VS2 20μM + NAC
ALIVE	93%	95%	43%	87%	38%	81%	44%	88%	25%	85%
APOPTOTIC	2%	2%	44%	9%	48%	13%	34%	7%	52%	9%
DEAD	4%	3%	14%	3%	15%	6%	22%	5%	23%	6%

Fig. 3 The induction of apoptosis is dependent on ROS production. The graphs show the results of the Annexin V apoptosis assays on A375 cells following treatment with VN and VS2 at concentrations of 10 and 20 μM, in the presence or absence of 5 mM NAC. The data represent the percentage of live, apoptotic (early + late) and dead cells after 24 hours (A) and 48 hours (B) from the administration of the two compounds.

Expression/activation of cell cycle regulators

In our previous work it was shown that the two compounds VN and VS2 determined a block in the progression of the cell cycle in A375 cells and, in particular, the compound VN led to a stop in the G2 phase, while the compound VS2 caused a stop in G1.[47] These results were confirmed by flow cytometry analysis as described in the ''Experimental'' section (data not shown).

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

To investigate the possible mechanisms of action of the VN and VS2 compounds we evaluated their effect on the levels of expression and/or activation of some key proteins that regulate the initiation and progression of the cell cycle. For this purpose, Western blot experiments were carried out using antibodies specific for the following proteins and/or their phosphorylated forms: ERK, phospho-ERK, phospho-Rb, $p21^{Cip1}$ and phospho-Cdc25C. The A375 cells were treated with the VN and VS2 compounds at concentrations of 10 and 20 μM, for 4 increasing time intervals (3, 6, 16 and 24 hours), then lysed and analyzed by Western blotting. The results of such experiments are shown in Fig. 4 and discussed as follows.

Phospho-ERK. Under physiological conditions, the ERK kinase is activated by the MAPK phosphorylation cascade, which is triggered by growth factors stimuli. Then, phospho-ERK translocates to the nucleus where it is able to activate different transcription factors that induce the expression of early cell cycle genes (such as cyclin D) and allow the transition of the cell from the G0 state to the G1 phase of the cycle.[66]

In our case, evaluating the phosphorylation levels of this kinase is of great importance because the A375 cell line used as an experimental model in this study carries the V600E mutation of the BRAF gene, as 60% of melanomas do.[52] B-Raf is a serine–threonine kinase which acts upstream of MEK and ERK in the MAPK pathway. The BRAF V600E mutation results in constitutively activated B-Raf kinase, making the MAPK cascade unceasingly active. Therefore, the ERK kinase is constantly phosphorylated, then activated, continuously signaling cells for entering the cycle. This is a feature common to many cancer cells, which never go into a quiescent state but continually enter a duplication cycle,[67] and it is one of the main features of the molecular genetics of melanoma.[3,7]

Our results, obtained from the analysis of the ERK and phosphorylated ERK kinase (ph-ERK) expression levels by Western blotting, are shown in Fig. 4, panels A and B. While the levels of ERK protein expression appeared pretty unvaried during the treatments with both VN and VS2 (Fig. 4, panel A), the levels of ph-ERK showed a gradual decrease over time in the A375 cell samples treated with the VN or VS2 compounds. Indeed, the ph-ERK levels dropped down to 20% compared to the control, after 24 hours of 20 μM treatment with either compound (Fig. 4, panel B). The decrease of ERK phosphorylation was more evident at short times particularly for the cells treated with VN, but it was very clear after 24 hours also for VS2 (Fig. 4, panel B). These data show that both the VN and VS2 compounds are able to counteract the constitutive activation of the MAPK cascade by inducing dephosphorylation of ERK. As a consequence, we can suppose this causes a lowering of the intensity of the mitotic signal continually triggered by the BRAF V600E mutation. This effect certainly contributes to the antiproliferative action exerted by the two compounds on A375 cells.[47] Counteracting the activation of the MAPK pathway, either by blocking the activity of the mutated BRAF protein, or by acting on the blockade of the downstream MEK-ERK kinases with specific inhibitors, is the principle on which the most recent target therapies successfully used against metastatic melanoma in the last years are based. In fact, they all lead to ERK dephosphorylation and arrest tumor growth.[3,8]

Our results on the MAPK pathway, though, contrast with those reported by other groups in the literature, which observed an increase in ERK phosphorylation induced by vanadium compounds in different *in vitro* models.[43,68,69] This could be strictly related to our specific melanoma model, carrying the V600E BRAF mutation, which makes the MAPK pathway constitutively active, resulting in basic ERK hyper-phosphorylation.

Phospho-Rb. We also analyzed the phosphorylation levels of the Rb protein, which plays a key role in controlling the transition from the G1 phase to the S phase of the cell cycle. The phosphorylation of Rb by the cyclinD1/CDK4/6 complex determines its deactivation and detachment from the transcription factor E2F, which in turn activates the transcription of target genes necessary for the cell cycle progression from the G1 to the S phase (cyclin E and cyclin A).[70] This pathway (Fig. 5) is one of the primary pathways involved in melanomagenesis.[3,7] The cyclin D1/CDK4/6 complex activity is indeed controlled by the $p16^{INK4A}$ tumor suppressor protein, encoded by the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene. CDKN2A is located on chromosome 9p21 and encodes two proteins, $p16^{INK4A}$ (including exons 1α, 2 and 3) and $p14^{INK4B}$ (a product of an alternative splicing that includes exons 1β and 2),[71] which are known to function as tumor suppressors. $p16^{INK4A}$ and $p14^{INK4B}$ are simultaneously altered in multiple tumors since most of their pathogenetic mutations occur in exon 2, which is encoded in both gene products. It represents one of the major genes involved in melanoma pathogenesis and predisposition. Our melanoma model, the A375 cell line, carries two CDKN2A mutations, resulting in the absence of both $p16^{INK4A}$ and $p14^{INK4B}$ tumor suppressor proteins. In particular, lack of $p16^{INK4A}$ results in increased phosphorylation/deactivation of Rb, allowing uncontrolled cell cycle progression from the G1 to the S phase. Analysis of Rb phosphorylation (ph-Rb) levels in A375 cells (Fig. 4, panel C) indicated, indeed, very high levels of ph-Rb in the untreated cells (ctrl in Fig. 4). But in the samples treated with VN and VS2 we observed a gradual and significant decrease of ph-Rb, reaching levels of 0% and 10%, respectively, after 16 hours of 20 μM VN and 24 hours of 10 μM VS2 treatments. These data indicate that the action of the two vanadium compounds promoted Rb dephosphorylation, presumably counteracting cell cycle progression and so contributing to the antiproliferative effect of VN and VS2 on melanoma cells. Such an effect could be due to the similarity of the vanadate ion to phosphate. Vanadate, in fact, could compete with the latter within the cell by replacing it, thus decreasing the phosphorylation levels of various substrates.[72,73] A similar effect has been previously described for the bis(acetylacetonato)oxidovanadium(IV) compound in HepG2 cells.[68]

In our melanoma model, inducing Rb dephosphorylation is a main feature because it counteracts the lack of the $p16^{INK4A}$ tumor suppressor and restores a sort of control over indiscriminate cell growth. For this reason, compounds like VN or VS2, which are able to decrease the uncontrolled phosphorylation of Rb, could be of great interest in the search for new therapeutic agents to be combined with specific targeted therapies, in order to counteract tumors carrying CDKN2A mutations/deletions.

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

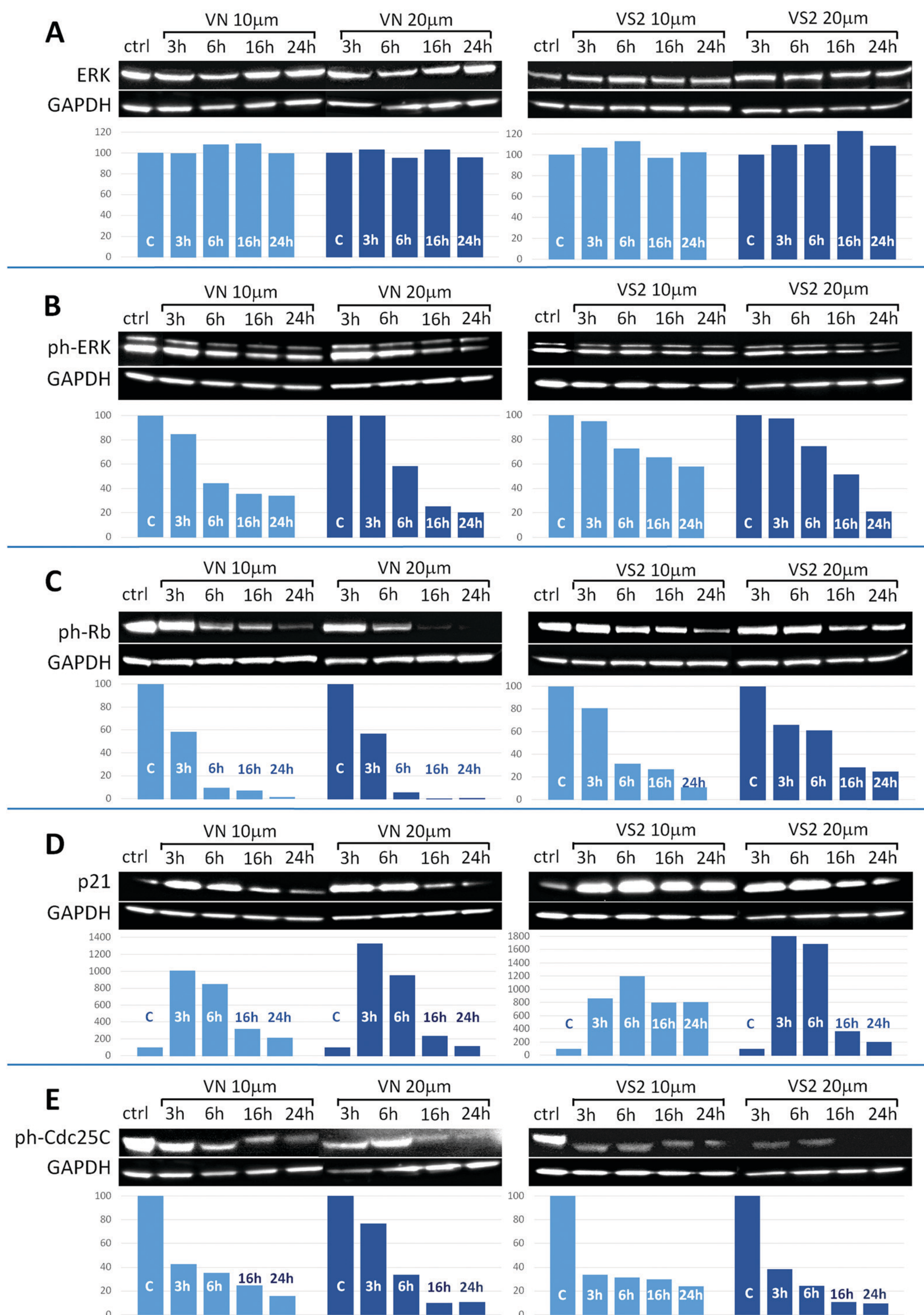


Fig. 4 Western blotting. A375 cells were treated with different concentrations of VN and VS2 (0, 10 and 20 μM) for different experimental times (3, 6, 16 and 24 hours). Cell lysates were resolved on SDS-PAGE and transferred on nitrocellulose filters, as described in the "Experimental" section. Filters were hybridized, respectively, with the following primary antibodies: anti ERK1/2 (ERK, panel A); anti-phospho-ERK1/2 (ph-ERK, panel B); anti phospho-Rb (ph-Rb, panel C); anti-p21^{Cip1} (p21, panel D); and anti phospho-Cdc25C (ph-Cdc25C, panel E). The levels of protein expression, quantified by the UVITEC mini HD6 imaging system, were analyzed and normalized against the levels of the GAPDH housekeeping gene expression with the UVITEC UVI-D1 software.

p21^{Cip1}. Oppositely to the two phosphorylated proteins described above, the expression of the CDK inhibitor p21^{Cip1} increased dramatically during the first few hours of treatment with the two compounds, rising 10–14 times for VN treated cells and up to 18 times in VS2 treated cells (Fig. 4, panel D). p21^{Cip1} is one of the main inhibitors of the cell cycle: it is able to block the formation of the CDK–cyclin complexes necessary for the transition from one phase to the next of the cell cycle.[74] Its expression

 This journal is © The Royal Society of Chemistry 2019

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

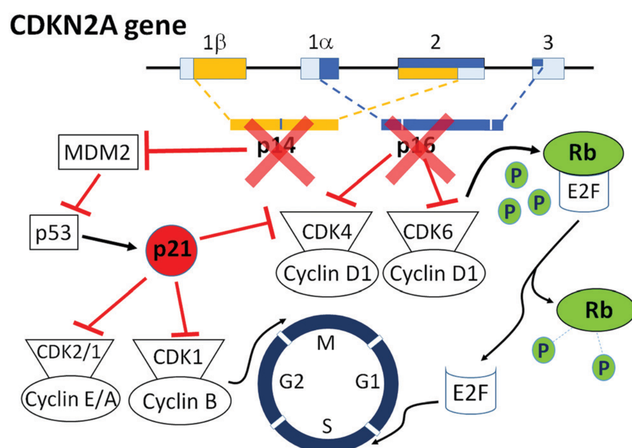


Fig. 5 CDKN2A gene pathway. Diagram of CDKN2A gene product ($p16^{INK4A}$ and $p14^{INK4B}$) pathways, showing their involvement in the regulation of the cell cycle progression. The A375 cell line carries two mutations in the CDKN2A gene, which expresses neither $p16^{INK4A}$ nor $p14^{INK4B}$ tumor suppressor proteins. A375 cells lack the negative regulation of the cell cycle exerted by the CDKN2A tumor suppressor gene.

is induced by the p53 protein, which can be activated by numerous factors, such as ROS production, cellular stress and DNA damage, and triggers various defense processes including blocking of cellular duplication and apoptosis.[75] The results observed in our experimental system indicate a strong induction of $p21^{Cip1}$ expression, which could be due to p53 stimulation triggered by the cellular stress following vanadium compound induced production of ROS and oxidative damage. This would explain the observed arrest of the cell cycle at different phases.

This feature is also very significant in our melanoma model, which is lacking the $p14^{INK4B}$ tumor suppressor protein (Fig. 5). $p14^{INK4B}$ normally blocks MDM2, which is responsible for p53 degradation, allowing p53 to exert its tumor control activity in several ways. One of these is to promote $p21^{Cip}$ expression, which is very low or absent in many tumors, including our melanoma model A375 (see Fig. 4, panel D, ctrl). Induction of $p21^{Cip}$ expression is therefore, another effective tool to counteract uncontrolled proliferation of our melanoma cells.

Phospho-Cdc25C. The last protein investigated is the Cdc25C phosphatase, a key element in the transition from phase G2 to phase M of the cell cycle. This phosphatase determines the activation of the CDK1–cyclin B complex by its dephosphorylation, which is necessary for the entry of the cell into mitosis.[76] In order for Cdc25C to be active, it must exhibit dephosphorylation on serine 216, thus allowing the transition from phase G2 to M. In our samples a decrease in phosphorylated Cdc25C (ph-Cdc25c) to 20–10% of the control was observed respectively in 10 or 20 μM VN/VS2 treated cells (Fig. 4, panel E), which suggests that this phosphatase is actually activated and therefore it is not affected by the action of the two vanadium compounds. The blockade in phase G2 observed in particular with the compound VN does not therefore seem to be related to a block of the activation of the Cdc25C phosphatase.

Based on our results, Fig. 6 shows a diagram schematizing a proposed mechanism of action for VN and VS2. VN enters the cell through ion channels, while VS2 enters by diffusion or endocytosis. Inside the cell both compounds induce dephosphorylation of different proteins, including phospho-ERK and phospho-Rb (left side). The ERK dephosphorylation effect,

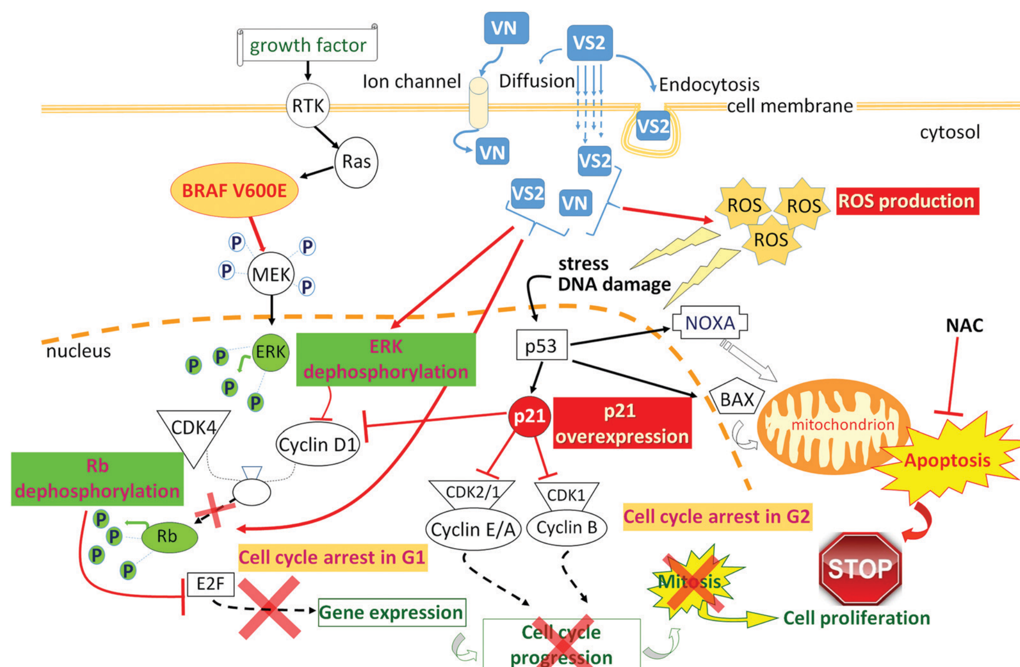


Fig. 6 Diagram of the VN and VS2 proposed mechanism of action (adapted from ref. 80).

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

probably due to competition of vanadate with phosphate ions, would lead to deactivation of the MAPK pathway, which was constitutively activated due to the V600E BRAF mutation. This provokes a block of ERK induced transcription of early cell cycle genes, such as cyclin D, resulting in a consequent antiproliferative effect.[66] Rb dephosphorylation, as well, would determine the inactivation of the E2F transcription factor, blocking the expression of the gene responsible for cell cycle progression to the S phase, thus inducing G1 phase arrest.[70] Moreover (right side), VN and VS2 both induce intracellular ROS production, causing cell stress and DNA damage. These events, as extensively described,[75] might stimulate p53 activity, which in turn promotes $p21^{Cip1}$ expression to block proliferation by inhibiting all CDK–cyclin complexes, and activates pro-apoptotic pathways.

Our results have excluded Cdc25C phosphatase from being involved in the G2 blocking of the cell-cycle. Therefore, in the immediate future we could analyze other proteins involved in the transition from phase G2 of the cell cycle to phase M, and others involved in the regulation of apoptosis, such as p53.

From a chemical point of view, the obtained results can be rationalized considering two vanadium properties already known in the literature. Vanadate(v), indicated with VN examined in this work, is structurally similar to phosphate and has been known for decades to inhibit enzymes that catalyze reactions of phosphorylation and dephosphorylation such as kinases and phosphatases.[77] It is therefore not surprising that the two vanadium compounds used interfere with the activity of proteins participating in molecular pathways that involve reactions of phosphorylation or dephosphorylation. The second property of vanadium which can explain some of the observed effects is the ability of its compounds to generate oxidative stress inside cells. Vanadium, similar to other essential metals such as copper and iron, has two easily accessible oxidation states, +IV and +V. As already mentioned above, the most stable oxidation state in the cellular environment (+IV) is able to give Fenton-like reactions leading to ROS formation. Finally, about the differences observed in the effects of the two compounds, VN and VS2, on A375 cells, we can hypothesize the following. The two compounds used cross the cell membrane using different mechanisms: VN, being anionic at physiological pH ($H_2VO_4^-$), uses the anionic channels, while VS2 being neutral and quite lipophilic is transported inside the cells by passive diffusion and/or endocytosis. This difference can result in different transport speeds and efficiencies and, consequently, different amounts of the two compounds can be found inside the cells. In particular, the VS2 compound is mostly stable and should remain, at least in part, unaltered inside the cells; VS2, however, can interact with proteins forming species of the VS2–protein type in which the metal is coordinated by histidine residues. On the contrary, VN, once inside the cells, is reduced by the cellular reducing agents to $V^{IV}O^{2+}$ and the latter is complexed by the cellular bioligands (proteins and binders with low molecular mass). These differences could explain, at least partially, the diverse effects observed with the two compounds. However it is not possible to exclude the partial oxidation of VS2 in the culture medium during the course of the experiments, as well as the partial reduction of VN and its complexation by some of the numerous components of the RPMI culture medium or added bovine fetal serum. Therefore, the pharmacological action of an administered V compound could be attributed, depending on the thermodynamic and redox stability in the cellular environment, to a series of species in the oxidation states +IV and +V in which also the bioligands of the cytosol can interact with vanadium.

Conclusions

For many years, vanadium compounds have been proposed in the literature as promising anticancer agents.[20] Initially, they were tested on the Ehrlich tumor, and subsequently on pancreas, breast, prostate, ovarian, and testis tumoral cell lines and on leukemia and glioblastoma cells. Only recently the cytotoxic activity of V complexes on melanoma cells was reported.[47,65]

The data presented in this study confirmed the antitumor activity of vanadium against malignant melanoma cells, highlighting the ability of the tested compounds to induce apoptosis through the production of ROS. They also demonstrated ERK dephosphorylation and consequent deactivation of the MAPK pathway by the two vanadium compounds in melanoma cells presenting the BRAF V600E mutation. MAPK pathway inactivation is consistent with the antiproliferative activity observed for these compounds in MM cells,[3,7] due to the resulting probable blocking of the cyclin D transcription, which would prevent the cell from entering the cycle. Furthermore, the dephosphorylation of Rb, resulting in E2F sequestration and blocking of the transcription of genes involved in the transition from the G1 to S cell cycle phases, is further evidence of a mechanism stopping the cycle in the G1 phase. Moreover, we can hypothesize that the induction of $p21^{Cip1}$ expression is linked to the probable activation of p53 due to the production of ROS and the derived cellular stress. p53 activation would lead to consequent inhibition of the Cdk/cyclin complexes by $p21^{Cip1}$, leading to cell cycle arrest at different stages and, on another front, to the induction of expression of pro-apoptotic proteins, such as BAX, NOXA, *etc.*, leading to apoptosis.

Finally, the results indicate that the pharmacological activity of vanadium cannot be attributed only to the administered compound but to several species in the oxidation states +IV and +V with V in the free or complexed form after the binding of the plasma or cell bioligands. We hope that these new insights could help to increase the knowledge (limited up to now) of the active species in the organism, which is one of the most important issues so that V compounds could be considered in the pipeline of pharmaceutical companies.[78,79]

We intend, in the immediate future, to analyze other proteins involved in the transition from phase G2 to phase M of the cell cycle, in order to clarify the mechanism leading to the G2 arrest observed following VN treatments, and other proteins involved in the regulation of apoptosis such as p53 and p53-induced pro-apoptotic proteins.

 This journal is © The Royal Society of Chemistry 2019

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by Fondazione di Sardegna (project FDS15Garribba) and Regione Autonoma della Sardegna (grant RASSR79857).

References

1 T. L. Diepgen and V. Mahler, The epidemiology of skin cancer, *Br. J. Dermatol.*, 2002, **146**, 1–6.

2 M. Berwick, D. B. Buller, A. Cust, R. Gallagher, T. K. Lee, F. Meyskens, S. Pandey, N. E. Thomas, M. B. Veierød and S. Ward, in *Melanoma Epidemiology and Prevention in Melanoma*, ed. H. L. Kaufman and J. M. Mehnert, Springer Int. Publ., Cham, 2016, pp. 17–49.

3 G. Palmieri, M. Ombra, M. Colombino, M. Casula, M. Sini, A. Manca, P. Paliogiannis, P. A. Ascierto and A. Cossu, Multiple Molecular Pathways in Melanomagenesis: Characterization of Therapeutic Targets, *Front. Oncol.*, 2015, **5**, 183.

4 S. Ribero, D. Glass and V. Bataille, Genetic epidemiology of melanoma, *Eur. J. Dermatol.*, 2016, **26**, 335–339.

5 AIOM, AIRTUM, Fondazione AIOM and PASSI, I numeri del cancro in Italia. I dati regionali. Intermedia Editore, Brescia, 2018.

6 G. Palmieri, M. Colombino, M. Casula, A. Manca, M. Mandalà and A. Cossu, for the Italian Melanoma Intergroup (IMI), Molecular Pathways in Melanomagenesis: What We Learned from Next-Generation Sequencing Approaches, *Curr. Oncol. Rep.*, 2018, **20**, 86.

7 G. Palmieri, M. Capone, M. L. Ascierto, G. Gentilcore, D. F. Stroncek, M. Casula, M. C. Sini, M. Palla, N. Mozzillo and P. A. Ascierto, Main roads to melanoma, *J. Transl. Med.*, 2009, **7**, 86.

8 D. J. L. Wong and A. Ribas, Targeted Theraphy for Melanoma, in *Melanoma*, ed. H. L. Kaufman and J. M. Mehnert, Springer International Publishing, Cham, 2016, pp. 251–262.

9 J. G. Greger, S. D. Eastman, V. Zhang, M. R. Bleam, A. M. Hughes, K. N. Smitheman, S. H. Dickerson, S. G. Laquerre, L. Liu and T. M. Gilmer, Combinations of BRAF, MEK, and PI3K/mTOR Inhibitors Overcome Acquired Resistance to the BRAF Inhibitor GSK2118436 Dabrafenib, Mediated by *NRAS* or *MEK* Mutations, *Mol. Cancer Ther.*, 2012, **11**, 909–920.

10 L. R. Strickland, H. C. Pal, C. A. Elmets and F. Afaq, Targeting drivers of melanoma with synthetic small molecules and phytochemicals, *Cancer Lett.*, 2015, **359**, 20–35.

11 P. A. Ascierto, K. Flaherty and S. Goff, Emerging Strategies in Systemic Therapy for the Treatment of Melanoma, *Am. Soc. Clin. Oncol. Educ. Book*, 2018, 751–758.

12 I. V. Fedorenko, G. T. Gibney, V. K. Sondak and K. S. M. Smalley, Beyond BRAF: where next for melanoma therapy?, *Br. J. Cancer*, 2014, **112**, 217–226.

13 M. Colombino, M. Sini, A. Lissia, V. De Giorgi, I. Stanganelli, F. Ayala, D. Massi, C. Rubino, A. Manca, P. Paliogiannis, S. Rossari, S. Magi, L. Mazzoni, G. Botti, M. Capone, M. Palla, P. A. Ascierto, A. Cossu and G. Palmieri, Discrepant alterations in main candidate genes among multiple primary melanomas, *J. Transl. Med.*, 2014, **12**, 117.

14 F. Arnesano and G. Natile, Mechanistic insight into the cellular uptake and processing of cisplatin 30 years after its approval by FDA, *Coord. Chem. Rev.*, 2009, **253**, 2070–2081.

15 N. P. E. Barry and P. J. Sadler, Exploration of the medical periodic table: towards new targets, *Chem. Commun.*, 2013, **49**, 5106–5131.

16 Z. Guo and P. J. Sadler, Metals in Medicine, *Angew. Chem., Int. Ed.*, 1999, **38**, 1512–1531.

17 S. Medici, M. Peana, V. M. Nurchi, J. I. Lachowicz, G. Crisponi and M. A. Zoroddu, Noble metals in medicine: Latest advances, *Coord. Chem. Rev.*, 2015, **284**, 329–350.

18 K. D. Mjos and C. Orvig, Metallodrugs in Medicinal Inorganic Chemistry, *Chem. Rev.*, 2014, **114**, 4540–4563.

19 B. Desoize, Metals and Metal Compounds in Cancer Treatment, *Anticancer Res.*, 2004, **24**, 1529–1544.

20 J. Costa Pessoa, S. Etcheverry and D. Gambino, Vanadium compounds in medicine, *Coord. Chem. Rev.*, 2015, **301–302**, 24–48.

21 J. Costa Pessoa and I. Tomaz, Transport of Therapeutic Vanadium and Ruthenium Complexes by Blood Plasma Components, *Curr. Med. Chem.*, 2010, **17**, 3701–3738.

22 A. Levina, D. C. Crans and P. A. Lay, Speciation of metal drugs, supplements and toxins in media and bodily fluids controls *in vitro* activities, *Coord. Chem. Rev.*, 2017, **352**, 473–498.

23 D. Rehder, Perspectives for vanadium in health issues, *Future Med. Chem.*, 2016, **8**, 325–338.

24 B. Mukherjee, B. Patra, S. Mahapatra, P. Banerjee, A. Tiwari and M. Chatterjee, Vanadium—an element of atypical biological significance, *Toxicol. Lett.*, 2004, **150**, 135–143.

25 D. Rehder, in *Interrelations between Essential Metal Ions and Human Diseases*, ed. A. Sigel, H. Sigel and R. K. O. Sigel, Springer, Netherlands, Dordrecht, 2013, pp. 139–169.

26 D. Rehder, Vanadium in health issues, *ChemTexts*, 2018, **4**, 20.

27 J. Kieler, A. Gromek and N. I. Nissen, Studies on the antineoplastic effect of vanadium salts, *Acta Chir. Scand.*, 1965, (Suppl. 343), 154–164.

28 P. Köpf-Maier and H. Köpf, Metallocene complexes: organometallic antitumor agents, *Drugs Future*, 1986, **11**, 297–319.

29 D. C. Crans, L. Yang, A. Haase and X. Yang, in *Metallo-Drugs: Development and Action of Anticancer Agents*, ed. A. Sigel, H. Sigel, E. Freisinger and R. K. O. Sigel, De Gruyter, Berlin, Boston, 2018, vol. 18, pp. 251–280.

30 S. Gomez-Ruiz, D. Maksimovic-Ivanic, S. Mijatovic and G. N. Kaluderovic, On the Discovery, Biological Effects, and Use of Cisplatin and Metallocenes in Anticancer Chemotherapy, *Bioinorg. Chem. Appl.*, 2012, 140284.

31 D. Rehder, The potentiality of vanadium in medicinal applications, *Future Med. Chem.*, 2012, **4**, 1823–1837.

View Article Online

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.

32 G. Mokdsi and M. M. Harding, Inhibition of human topoisomerase II by the antitumor metallocenes, *J. Inorg. Biochem.*, 2001, **83**, 205–209.

33 D. Sanna, V. Ugone, G. Micera, P. Buglyó, L. Bíró and E. Garribba, Speciation in human blood of Metvan, a vanadium based potential anti-tumor drug, *Dalton Trans.*, 2017, **46**, 8950–8967.

34 R. K. Narla, Y. Dong, O. J. D'Cruz, C. Navara and F. M. Uckun, Bis(4,7-dimethyl-1,10-phenanthroline) Sulfatooxovanadium(IV) as a Novel Apoptosis-inducing Anticancer Agent, *Clin. Cancer Res.*, 2000, **6**, 1546–1556.

35 R. K. Narla, Y. Dong, D. Klis and F. M. Uckun, Bis(4,7-dimethyl-1,10-phenanthroline) Sulfatooxovanadium(IV) as a Novel Antileukemic Agent with Matrix Metalloproteinase Inhibitory Activity, *Clin. Cancer Res.*, 2001, **7**, 1094–1101.

36 D. A. Barrio and S. B. Etcheverry, Potential Use of Vanadium Compounds in Therapeutics, *Curr. Med. Chem.*, 2010, **17**, 3632–3642.

37 I. E. León, P. Díez, E. J. Baran, S. B. Etcheverry and M. Fuentes, Decoding the anticancer activity of VO-clioquinol compound: the mechanism of action and cell death pathways in human osteosarcoma cells, *Metallomics*, 2017, **9**, 891–901.

38 I. E. León, P. Díez, S. B. Etcheverry and M. Fuentes, Deciphering the effect of an oxovanadium(iv) complex with the flavonoid chrysin (VOChrys) on intracellular cell signalling pathways in an osteosarcoma cell line, *Metallomics*, 2016, **8**, 739–749.

39 E. G. Ferrer, M. V. Salinas, M. J. Correa, L. Naso, D. A. Barrio, S. B. Etcheverry, L. Lezama, T. Rojo and P. A. M. Williams, Synthesis, characterization, antitumoral and osteogenic activities of quercetin vanadyl(IV) complexes, *JBIC, J. Biol. Inorg. Chem.*, 2006, **11**, 791–801.

40 D. C. Crans, H. LaRee, G. Cardiff and B. I. Posner, in *Essential Metals in Medicine: Therapeutic Use and Toxicity of Metal Ions in the Clinic*, ed. P. L. Carver, De Gruyter, Berlin, Boston, 2019, vol. 19, pp. 203–230.

41 I. E. Leon, J. F. Cadavid-Vargas, A. L. Di Virgilio and S. B. Etcheverry, Vanadium, Ruthenium and Copper Compounds: A New Class of Nonplatinum Metallodrugs with Anticancer Activity, *Curr. Med. Chem.*, 2017, **24**, 112–148.

42 E. Kioseoglou, S. Petanidis, C. Gabriel and A. Salifoglou, The chemistry and biology of vanadium compounds in cancer therapeutics, *Coord. Chem. Rev.*, 2015, **301-302**, 87–105.

43 J.-X. Wu, Y.-H. Hong and X.-G. Yang, Bis(acetylacetonato)-oxidovanadium(IV) and sodium metavanadate inhibit cell proliferation *via* ROS-induced sustained MAPK/ERK activation but with elevated AKT activity in human pancreatic cancer AsPC-1 cells, *J. Biol. Inorg. Chem.*, 2016, **21**, 919–929.

44 Z. Zhang, F. Chen, C. Huang and X. Shi, Vanadate Induces G2/M Phase Arrest in p53-Deficient Mouse Embryo Fibroblasts, *J. Environ. Pathol., Toxicol. Oncol.*, 2002, **21**, 9.

45 S. Markopoulou, E. Kontargiris, C. Batsi, T. Tzavaras, I. Trougakos, D. A. Boothman, E. S. Gonos and E. Kolettas, Vanadium-induced apoptosis of HaCaT cells is mediated by c-fos and involves nuclear accumulation of clusterin, *FEBS J.*, 2009, **276**, 3784–3799.

46 A. P. Gonçalves, A. Videira, P. Soares and V. Máximo, Orthovanadate-induced cell death in RET/PTC1-harboring cancer cells involves the activation of caspases and altered signaling through PI3K/Akt/mTOR, *Life Sci.*, 2011, **89**, 371–377.

47 C. Rozzo, D. Sanna, E. Garribba, M. Serra, A. Cantara, G. Palmieri and M. Pisano, Antitumoral effect of vanadium compounds in malignant melanoma cell lines, *J. Inorg. Biochem.*, 2017, **174**, 14–24.

48 D. J. Giard, S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik and W. P. Parks, *In Vitro* Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors, *J. Natl. Cancer Inst.*, 1973, **51**, 1417–1423.

49 H.-Y. Kim, H. Lee, S.-H. Kim, H. Jin, J. Bae and H.-K. Choi, Discovery of potential biomarkers in human melanoma cells with different metastatic potential by metabolic and lipidomic profiling, *Sci. Rep.*, 2017, **7**, 8864.

50 J. M. Kozlowski, I. R. Hart, I. J. Fidler and N. Hanna, A Human Melanoma Line Heterogeneous With Respect to Metastatic Capacity in Athymic Nude Mice234, *J. Natl. Cancer Inst.*, 1984, **72**, 913–917.

51 L. A. von Schuckmann, M. C. B. Hughes, R. Ghiasvand, M. Malt, J. C. van der Pols, V. L. Beesley, K. Khosrotehrani, B. M. Smithers and A. C. Green, Risk of Melanoma Recurrence After Diagnosis of a High-Risk Primary Tumor, *JAMA Dermatol.*, 2019, **155**, 688–693.

52 F. G. Cordaro, A. L. De Presbiteris, R. Camerlingo, N. Mozzillo, G. Pirozzi, E. Cavalcanti, A. Manca, G. Palmieri, A. Cossu and G. Ciliberto, Phenotype characterization of human melanoma cells resistant to dabrafenib, *Oncol. Rep.*, 2017, **38**, 2741–2751.

53 P. Buglyó, T. Kiss, E. Kiss, D. Sanna, E. Garribba and G. Micera, Interaction between the low molecular mass components of blood serum and the VO(IV)–DHP system (DHP = 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone, *J. Chem. Soc., Dalton Trans.*, 2002, 2275–2282.

54 M. Rangel, A. Leite, M. João Amorim, E. Garribba, G. Micera and E. Lodyga-Chruscinska, Spectroscopic and Potentiometric Characterization of Oxovanadium(IV) Complexes Formed by 3-Hydroxy-4-Pyridinones. Rationalization of the Influence of Basicity and Electronic Structure of the Ligand on the Properties of $V^{IV}O$ Species in Aqueous Solution, *Inorg. Chem.*, 2006, **45**, 8086–8097.

55 D. Rehder, *Bioinorganic vanadium chemistry*, John Wiley & Sons, 2008.

56 M. Pisano, I. De Paola, V. Nieddu, I. Sassu, S. Cossu, G. Galleri, A. Del Gatto, M. Budroni, A. Cossu, M. Saviano, G. Palmieri, L. Zaccaro and C. Rozzo, *In Vitro* Activity of the $\alpha v\beta 3$ Integrin Antagonist RGDechi-hCit on Malignant Melanoma Cells, *Anticancer Res.*, 2013, **33**, 871–879.

57 I. E. León, N. Butenko, A. L. Di Virgilio, C. I. Muglia, E. J. Baran, I. Cavaco and S. B. Etcheverry, Vanadium and cancer treatment: Antitumoral mechanisms of three oxidovanadium(IV) complexes on a human osteosarcoma cell line, *J. Inorg. Biochem.*, 2014, **134**, 106–117.

58 I. E. Leon, A. L. Di Virgilio, V. Porro, C. I. Muglia, L. G. Naso, P. A. M. Williams, M. Bollati-Fogolin and S. B. Etcheverry,

 This journal is © The Royal Society of Chemistry 2019

Antitumor properties of a vanadyl(IV) complex with the flavonoid chrysin [VO(chrysin)$_2$EtOH]$_2$ in a human osteosarcoma model: the role of oxidative stress and apoptosis, *Dalton Trans.*, 2013, **42**, 11868–11880.
59 J. Wang, X. Huang, K. Zhang, X. Mao, X. Ding, Q. Zeng, S. Bai, Y. Xuan and H. Peng, Vanadate oxidative and apoptotic effects are mediated by the MAPK-Nrf2 pathway in layer oviduct magnum epithelial cells, *Metallomics*, 2017, **9**, 1562–1575.
60 M. Zafarullah, W. Q. Li, J. Sylvester and M. Ahmad, Molecular mechanisms of N-acetylcysteine actions, *Cell. Mol. Life Sci.*, 2003, **60**, 6–20.
61 D. Sanna, J. Palomba, G. Lubinu, P. Buglyó, S. Nagy, F. Perdih and E. Garribba, Role of Ligands in the Uptake and Reduction of V(V) Complexes in Red Blood Cells, *J. Med. Chem.*, 2019, **62**, 654–664.
62 D. Sanna, V. Ugone, A. Fadda, G. Micera and E. Garribba, Behavior of the potential antitumor $V^{IV}O$ complexes formed by flavonoid ligands. 3. Antioxidant properties and radical production capability, *J. Inorg. Biochem.*, 2016, **161**, 18–26.
63 A. Bijelic, M. Aureliano and A. Rompel, Polyoxometalates as Potential Next-Generation Metallodrugs in the Combat Against Cancer, *Angew. Chem., Int. Ed.*, 2019, **58**, 2980–2999.
64 R. M. C. Gândara, S. S. Soares, H. Martins, C. Gutiérrez-Merino and M. Aureliano, Vanadate oligomers: *In vivo* effects in hepatic vanadium accumulation and stress markers, *J. Inorg. Biochem.*, 2005, **99**, 1238–1244.
65 M. Strianese, A. Basile, A. Mazzone, S. Morello, M. C. Turco and C. Pellecchia, Therapeutic potential of a pyridoxal-based vanadium(IV) complex showing selective cytotoxicity for cancer *versus* healthy cells, *J. Cell. Physiol.*, 2013, **228**, 2202–2209.
66 M. Burotto, V. L. Chiou, J.-M. Lee and E. C. Kohn, The MAPK pathway across different malignancies: A new perspective, *Cancer*, 2014, **120**, 3446–3456.
67 D. Hanahan and R. A. Weinberg, Hallmarks of Cancer: The Next Generation, *Cell*, 2011, **144**, 646–674.
68 Y. Fu, Q. Wang, X.-G. Yang, X.-D. Yang and K. Wang, Vanadyl bisacetylacetonate induced G1/S cell cycle arrest *via* high-intensity ERK phosphorylation in HepG2 cells, *J. Biol. Inorg. Chem.*, 2008, **13**, 1001–1009.
69 J. Rivadeneira, A. L. D. Virgilio, D. A. Barrio, C. I. Muglia, L. Bruzzone and S. B. Etcheverry, Cytotoxicity of a Vanadyl(IV) Complex with a Multidentate Oxygen Donor in Osteoblast Cell Lines in Culture, *Med. Chem.*, 2010, **6**, 9–23.
70 C. Giacinti and A. Giordano, RB and cell cycle progression, *Oncogene*, 2006, **25**, 5220–5227.
71 J. Gil and G. Peters, Regulation of the INK4b–ARF–INK4a tumour suppressor locus: all for one or one for all, *Nat. Rev. Mol. Cell Biol.*, 2006, **7**, 667–677.
72 E. Irving and A. W. Stoker, Vanadium Compounds as PTP Inhibitors, *Molecules*, 2017, **22**, 2269.
73 I. Sánchez-Lombardo, S. Alvarez, C. C. McLauchlan and D. C. Crans, Evaluating transition state structures of vanadium–phosphatase protein complexes using shape analysis, *J. Inorg. Biochem.*, 2015, **147**, 153–164.
74 Y. Xiong, G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi and D. Beach, p21 is a universal inhibitor of cyclin kinases, *Nature*, 1993, **366**, 701–704.
75 E. R. Kastenhuber and S. W. Lowe, Putting p53 in Context, *Cell*, 2017, **170**, 1062–1078.
76 J. Rudolph, Cdc25 Phosphatases: Structure, Specificity, and Mechanism, *Biochemistry*, 2007, **46**, 3595–3604.
77 L. C. Cantley, L. Josephson, R. Warner, M. Yanagisawa, C. Lechene and G. Guidotti, Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle, *J. Biol. Chem.*, 1977, **252**, 7421–7423.
78 T. Scior, A. Guevara-Garcia, P. Bernard, Q.-T. Do, D. Domeyer and S. Laufer, Are Vanadium Compounds Drugable? Structures and Effects of Antidiabetic Vanadium Compounds: A Critical Review, *Mini-Rev. Med. Chem.*, 2005, **5**, 995–1008.
79 T. Scior, J. A. Guevara-Garcia, Q.-T. Do, P. Bernard and S. Laufer, Why Antidiabetic Vanadium Complexes are Not in the Pipeline of ''Big Pharma'' Drug Research? A Critical Review, *Curr. Med. Chem.*, 2016, **23**, 2874–2891.
80 C. Rozzo, M. Fanciulli, C. Fraumene, A. Corrias, T. Cubeddu, I. Sassu, S. Cossu, V. Nieddu, G. Galleri, E. Azara, M. A. Dettori, D. Fabbri, G. Palmieri and M. Pisano, Molecular changes induced by the curcumin analogue D6 in human melanoma cells, *Mol. Cancer*, 2013, **12**, 37.

Published on 21 August 2019. Downloaded by CNR on 9/6/2019 10:56:49 AM.