Dalton Transactions

RSC Publishing

PAPER

Antitumor properties of a vanadyl(IV) complex with the flavonoid chrysin $[VO(chrysin)_2EtOH]_2$ in a human osteosarcoma model: the role of oxidative stress and apoptosis†

Cite this: *Dalton Trans.*, 2013, **42**, 11868

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Received 26th February 2013,
Accepted 21st May 2013

DOI: 10.1039/c3dt50524c

www.rsc.org/dalton

Flavonoids, a polyphenolic compound family, and the vanadium compounds have interesting biological, pharmacological, and medicinal properties. We report herein the antitumor actions of the complex $[VO(chrysin)_2EtOH]_2$ (VOchrys) on the MG-63 human osteosarcoma cell line. Oxovanadium(IV), chrysin and VOchrys caused a concentration-dependent inhibition of cell viability. The complex was the strongest antiproliferative agent ($p < 0.05$). Cytotoxicity and genotoxicity studies also showed a concentration effect. Reactive oxygen species (ROS) and the alterations in the GSH/GSSG ratio underlie the main mechanisms of action of VOchrys. Additions of ROS scavengers (vitamin C plus vitamin E) or GSH to the viability experiments demonstrated beneficial effects ($p < 0.01$). Besides, the complex triggered apoptosis, disruption of the mitochondria membrane potential (MMP), increased levels of caspase 3 and DNA fragmentation measured by the sub-G1 peak in cell cycle arrest experiments ($p < 0.01$). Collectively, VOchrys is a cell death modulator and a promissory complex to be used in cancer treatments.

Introduction

Flavonoids are polyphenolic compounds widely distributed in the plant kingdom.[1] Different chemical classes of flavonoids have been established by IUPAC.[2]

These polyphenolic benzo-γ-pyrone compounds are of great interest for their potential pharmacological effects on animals and human beings: antiallergenic, antiviral, antiinflammatory, antitumor, and vasodilating actions.[3–5]

The medical applications of flavonoid derivatives arise mainly from their antioxidative and cytoprotective properties.[6]

A wide variety of diseases involve free-radical mediated damage by increasing the oxidative stress in the organism.[7,8] Nevertheless, the underlying mechanisms are very complex. For instance, some antioxidants display a pro-oxidant effect depending on the concentration and environment in which they act.[9]

On the other hand, the physiological properties of flavonoids are enhanced upon complexation with metal ions.[10]

Chrysin, 5,7-dihydroxy-2-phenyl-4*H*-chromen-4-one (Fig. 1), is a naturally occurring flavone extracted from the blue passion flower and from the honeycomb.[11] The antitumor effects of chrysin have been previously reported in several tumoral cell lines.[12–14]

Besides, we have previously reported that in the UMR106 rat osteosarcoma cell line, chrysin caused a decrease of cell proliferation of *ca.* 40% at 25 μM, while its effect on the non-transformed osteoblasts MC3T3-E1 was less than 20% at the same concentration.[1]

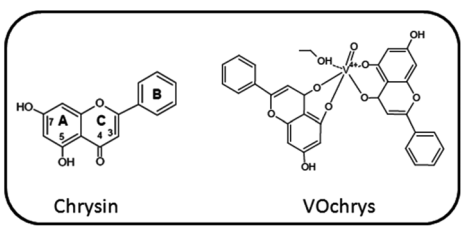

Fig. 1 Chemical structure of Chrysin, 5,7-dihydroxy-2-phenyl-4*H*-chromen-4-one and schematic structure of VOchrys (derived from ref. 1).

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c3dt50524c

Coordination complexes of transition metals and chrysin are scarcely reported in the literature and so far their biological properties have not been examined.

On the other hand, vanadium is an ultra-trace element with interesting biological and pharmacological properties present in higher plants and animals.[15,16] Greater quantities of vanadium are detected in the liver, kidney and especially in bones.[15]

The biological actions of vanadium convert its compounds into possible therapeutic agents to be used in the treatment of a number of diseases. In particular, vanadate(v) and vanadyl(iv) derivatives show insulin-mimetic/antidiabetic activity, growth factor and osteogenic actions,[17,18] antitumor properties,[19] cardioprotective actions[20] and neurologic effects.[21]

Vanadium compounds have been considered as a new class of metal-based antitumor agents in the last few decades.[19,22,23] Several vanadium compounds have different effects on cell viability.[23]

Different mechanisms have been described to explain the inhibition of cell cycle or the induction of tumor cell death by vanadium derivatives. In particular, the generation of ROS may cause a series of cellular effects such as DNA cleavage and oxidative damage of different cellular components and finally triggers apoptosis and cell cycle arrest.

The present study deals with the effects of an oxovanadium(iv) complex with the flavonoid chrysin, $[VO(chrysin)_2EtOH]_2$, (VOchrys) (for a schematic representation of the complex see Fig. 1) on the human osteosarcoma cell line MG-63.

We have investigated the action of this compound on cell viability and the putative mechanisms involved in its antiproliferative effects. In particular, we focus our attention on the role of oxidative stress and the cytotoxicity and genotoxicity actions of this complex.

Results and discussion

Synthesis and identification of VOchrys

VOchrys was synthetized according to Naso *et al.*[1] The method is briefly described in the Materials and Methods sections.

The obtained powder sample of the complex was identified by FTIR and the main vibrations of the organic moiety were: 1631 s; 1596 s; 1521 vs; 1428 s; 1350 s; 1161 vs. The vibration corresponding to the ν(V=O) with a medium intensity was placed at 968 cm^{-1}.

On the other hand, from the physicochemical characterizations of VOchrys derived from the spectroscopic studies of ref. 1, the schematic structure proposed for the complex can be seen in Fig. 1. Besides, from the EPR data, it was assumed that in the proposed complex structure the axial positions were occupied by the oxygen atom of V=O and the other axial position by a solvent molecule. The equatorial positions are occupied by two organic ligand molecules. In solution, it can be assumed that the main species is VOL_2, taking into account the L/M ratio.[1]

Effect of chemical complexation on cell viability

Fig. 2 shows the effects of the flavonoid chrysin, the vanadyl(iv) cation and the complex VOchrys on the proliferation of MG-63 osteosarcoma cells of human origin. As can be seen, vanadyl(iv) cation provoked an inhibitory effect only at 100 μM, while chrysin and the complex impaired cell viability from 10 μM ($p < 0.01$). Nevertheless, the antiproliferative action of the complex is much stronger than that of the free flavonoid in the whole range of the studied concentrations ($p < 0.05$), demonstrating an improvement of the antitumor action through the complexation of the chrysin with vanadyl(iv). This is also evident from the IC_{50} values in MG-63: for VOchrys, *ca.* 16 μM, while for chrysin and vanadyl(iv) cations the IC_{50} are >100 μM. This result demonstrates that VOchrys is the strongest antiproliferative agent.

Comparison of VOchrys cytotoxicity in osteoblast cell lines

The deleterious effect of VOchrys on another tumor osteoblast cell line of murine origin (UMR106) and on a non-transformed osteoblast cell line (MC3T3-E1) was previously reported by our group.[1] Fig. 1 (ESI†) displays the comparative effects of VOchrys on the three osteoblast cell lines. The complex produced its antiproliferative effects with the following increase of potency: MC3T3-E1 < UMR106 < MG-63, with statistically significant differences between the three cell lines ($p < 0.05$).

As a whole, these results indicate that VOchrys is a potentially good candidate for further evaluation of its mechanisms of action since it is less toxic to the non-transformed osteoblasts and is highly deleterious for the rat and human osteosarcoma cells.

Moreover, we have also studied the effects of VOchrys on the cellular morphology of the MG-63 cell line. Numerous publications, based on the alterations induced by different chemical compounds on cellular cultures, have shown that these

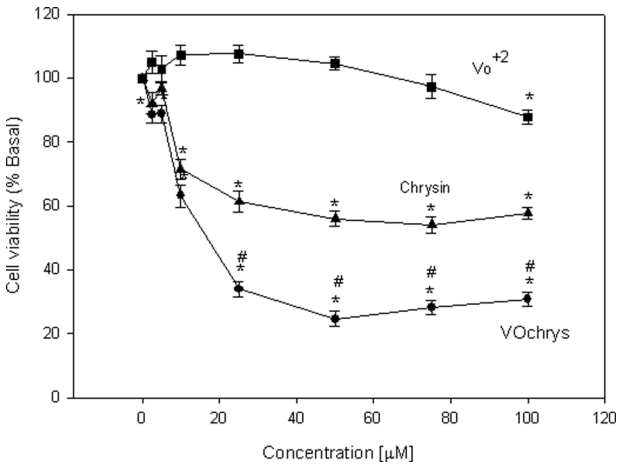

Fig. 2 Effects of VOchrys on MG-63 human osteosarcoma cell line viability evaluated by crystal violet. Cells were incubated in serum-free DMEM alone (control) or with different concentrations of the complex at 37 °C for 24 h. The results are expressed as the percentage of the basal level and represent the mean ± SEM ($n = 18$). * significant difference in comparison with the basal level ($p < 0.01$). # significant differences between the complex and the ligand ($p < 0.01$).

changes are good markers to infer the type of cell death (apoptosis or necrosis).[24,25]

Fig. 2 (ESI†) shows the morphological features of MG-63 osteoblast like cells and the effects of 10 and 25 μM of VOchrys.

MG-63 cells showed fibroblastic features with very well stained cytoplasm and oval nuclei, and the monolayer exhibited multiple connections between cells. The complex caused a slight change at 10 μM and the deleterious actions increased with the complex concentration as could be seen for 25 μM, where a marked decrease in the number of cells per field was observed with pronounced alterations in the cytoplasm and the nuclei. These results correlated with those of the viability study as previously reported for other vanadium derivatives.[24–26]

Cytotoxicity studies

In order to get a deeper insight into the antiproliferative effects of VOchrys, the cytotoxicity of the complex to two relevant organelles of the cells such as lysosomes and mitochondria was investigated through the neutral red (NR) uptake and the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively.

The NR technique is used to measure the growth of a population of cultured cells: viable cells take up the NR dye and transport it to a specific cellular compartment, the lysosomes. The uptake, transport, and storage of the NR dye require energy, as well as intact cellular and lysosomal structures.[27] The metabolically active lysosomes display the capacity for uptake of the NR dye. A loss of lysosomal activity, indicated by a decrease in the uptake of NR, was observed when MG-63 cells were exposed to increasing concentrations of VOchrys (Fig. 3A). The data presented herein show a cytotoxic effect of VOchrys in a concentration-dependent manner from 2.5 to 25 μM with statistically significant differences *versus* control conditions (without complex addition) ($p < 0.01$) (Fig. 3A). The inhibitory effect showed a *plateau* in the higher concentration range (50–100 μM).

The alteration in the energetic cell metabolism can be determined by the MTT assay. This technique measures the ability of the mitochondrial succinic dehydrogenases to reduce the methyl tetrazolium salt (MTT).[28] Fig. 3B shows the effects of VOchrys on the mitochondria metabolism of MG-63 osteosarcoma cells. As can be seen, a concentration related inhibition was observed from 10 to 25 μM with statistically significant differences *versus* the basal condition ($p < 0.01$). After the latter concentration a constant inhibition was observed for the high concentration range (50–100 μM) similar to the effect of the complex on the lysosome metabolism.

These two tests demonstrate the cytotoxic actions of the complex which affected the normal activity of the lysosomes and the mitochondria, contributing in this way to the deleterious effect of VOchrys on the tumoral cell line MG-63. It can therefore be suggested that there is a probable toxic interaction between oxidative stress and lysosomal and mitochondrial metabolism disruption.

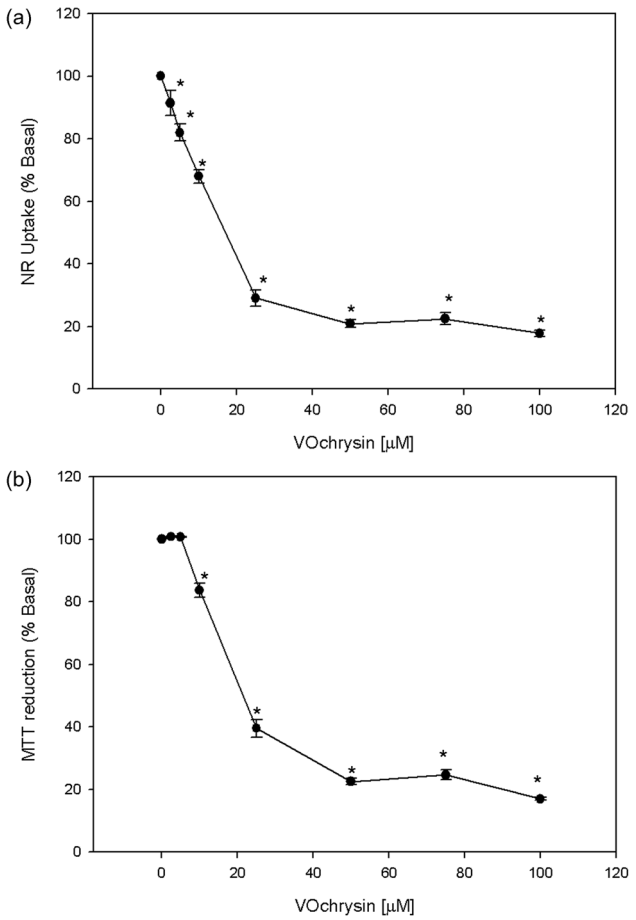

Fig. 3 (a) NR uptake by MG-63 osteosarcoma cells in culture. Tumoral cells were incubated with different concentrations of VOchrys for 24 h at 37 ° C. After incubation, cell viability was determined by the uptake of NR. The dye taken up by the cells was extracted and the absorbance read at 540 nm. Results are expressed as % basal and represent the mean ± SEM, $n = 18$, $^*p < 0.01$. (b) Evaluation of the mitochondrial succinate dehydrogenase activity by the MTT assay in MG-63 cells in culture. Osteosarcoma cells were incubated with different concentrations of VOchrys at 37 ° C for 24 h. After incubation, cell viability was determined by the MTT assay. Results are expressed as % basal and represent the mean ± SEM, $n = 18$, $^*p < 0.01$.

Genotoxicity study

The genotoxic effects of VOchrys were investigated through the increase of micronuclei (MN) frequency and the induction of DNA damage.[29]

The effect of VOchrys on MN induction in binucleated cells (which are those selected for this assay) can be observed from Fig. 4A. In MG-63 cells, the complex induced micronuclei formation in a dose response manner ($p < 0.01$) from 2.5 to 5 μM. Besides, bleomycin (1 μg mL^{-1}) was used as a positive control.[30]

The single cell gel electrophoresis (SCGE) or Comet assay is another test used for the investigation of genotoxicity. It detects single and double strand DNA breaks. Sites where excision and repairs have occurred are detected under alkaline conditions.[31] For VOchrys we evaluated the tail moment parameter which is defined as the tail length × DNA amount in

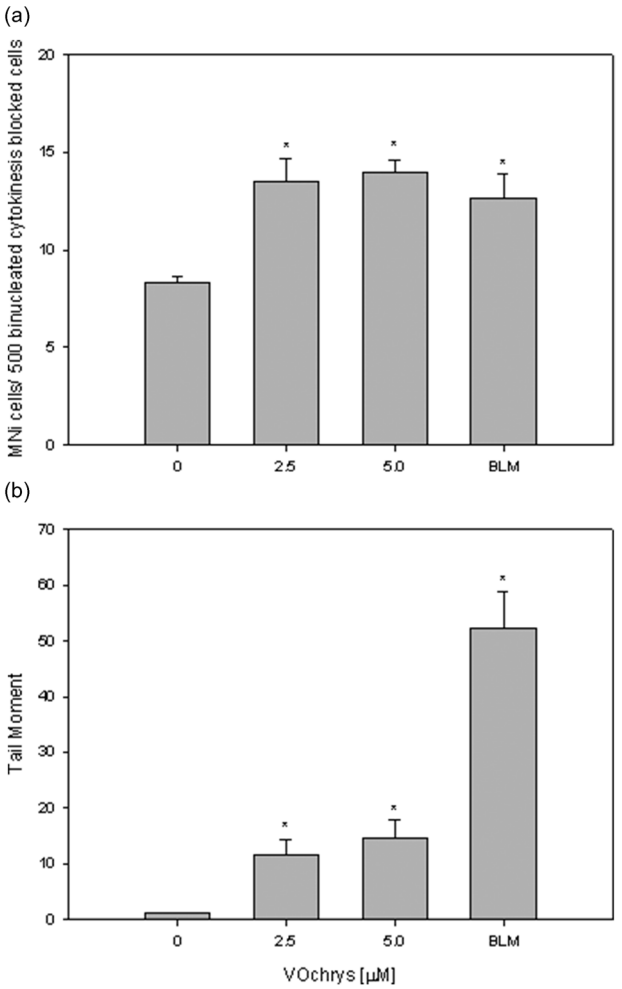

Fig. 4 Genotoxicity of VOchrys on MG-63 tumoral cells. (a) MN assay: micronuclei induction in MG-63 cells after 24 h exposure to VOchrys. * Significant difference at $p < 0.01$. BLM stands for Bleomycin used as a positive control. (b) Induction of DNA damage by VOchrys in MG-63 human osteosarcoma cell line determined by SCGE (Comet assay). DNA damage was evaluated by the Tail Moment. After incubation with VOchrys for 24 h, cells were lysed and DNA fragments were processed by electrophoresis. After that, the nuclei were stained and analyzed. Results are expressed as mean ± SEM ($n = 150$), *$p < 0.001$. BLM stands for Bleomycin used as a positive control.

the tail. The distance of DNA migration is used to measure the extent of DNA damage. However, if DNA damage is relatively high, the tail increases in fluorescent staining intensity but not in length.[32] Thus, for these reasons it is useful to use the tail moment as a genotoxic endpoint. As it is shown in Fig. 4B, VOchrys produced a significant genotoxic effect in MG-63 cells from 2.5 to 5 μM ($p < 0.001$). From 10 to 25 μM the genotoxic effect is less pronounced (data not shown). The decrease in DNA damage as the complex concentration increases may be due to overt cytotoxicity exerted on this cell line.

Altogether, these results suggest that VOchrys induced single and double strand DNA breaks in MG-63 cells, leading to a positive result in the Comet assay and induction in micronuclei frequency.

According to our findings, DNA damage was also reported in human blood leukocytes exposed to vanadium oxides *in vitro*. It was observed that the genotoxic effect of vanadium can be produced by any of its three oxidation states. However, vanadium(IV) induces double-strand breaks, and it is believed that these lesions are linked with the formation of structural chromosomal aberrations.[33] Moreover, we have previously reported the genotoxic effect of a complex of vanadyl(IV) cation with oxodiacetate employing the Comet assay in tumoral Caco-2 cells. This action agreed with the effect on plasmidic DNA causing single and double strand cleavage.[26] We have also studied the genotoxicity of hydroxylamido–amino acid complexes of oxovanadium(V) in osteoblast-like cell lines, showing an effect dependent on the cell line.[34] On the other hand, *in vivo* experiments showed the genotoxicity of tetravalent vanadium. Vanadyl sulphate in male CD1 mice produced an increase in the incidence of micronucleated blood reticulocytes and bone marrow polychromatic erythrocytes, in addition to DNA lesions detectable by the Comet assay.[35]

Mechanism of action

The putative cell death mechanisms triggered by VOchrys were investigated through the determination of the oxidative stress (by means of reactive oxygen species (ROS) production), GSH levels as well as the redox couple GSH/GSSG. Moreover, an exhaustive study of apoptosis and cell cycle arrest was also performed.

Oxidative stress. Vanadium compounds exert their toxic effects, at least in part, through the generation of oxidative stress.[19,24,34,36–38]

For a better understanding of the possible mechanism involved in the cytotoxicity of VOchrys in MG-63 cells, we evaluated the effect of this complex on oxidative stress through the oxidation of the probe DHR-123 and the ratio GSH/GSSG.

DHR-123 is a mitochondria-associated probe that selectively reacts with hydrogen peroxide.[36,39] The results of the incubation of MG-63 osteosarcoma cells on the induction of ROS can be observed in Fig. 5A. The complex increased ROS production in a dose-dependent manner with statistical differences in the range of 10 to 100 μM ($p < 0.01$). At the highest tested concentration (100 μM) the complex caused *ca.* 331% of ROS increment over basal. Results previously reported by our group have also shown that ROS formation was also dependent on the cellular type.[24,26] The increase in ROS levels has been associated with cell death or membrane injury. It has been reported that high levels of ROS can induce apoptosis by activating either the ER stress-mediated apoptotic pathway or the mitochondrial-mediated apoptotic pathway or both. The elevation of cytosolic Ca^{2+}, due to ER stress, may trigger mitochondrial permeability transition pore opening, cytochrome *c* release, caspase cascade activation, and apoptosis.[40,41]

This finding triggers new proliferation studies to determine the effect of ROS level on the antiproliferative action of VOchrys. When the cells were incubated with increasing doses of the complex in the presence of a mixture of 50 μM of vitamin C plus 50 μM of vitamin E (ROS scavengers), a partial

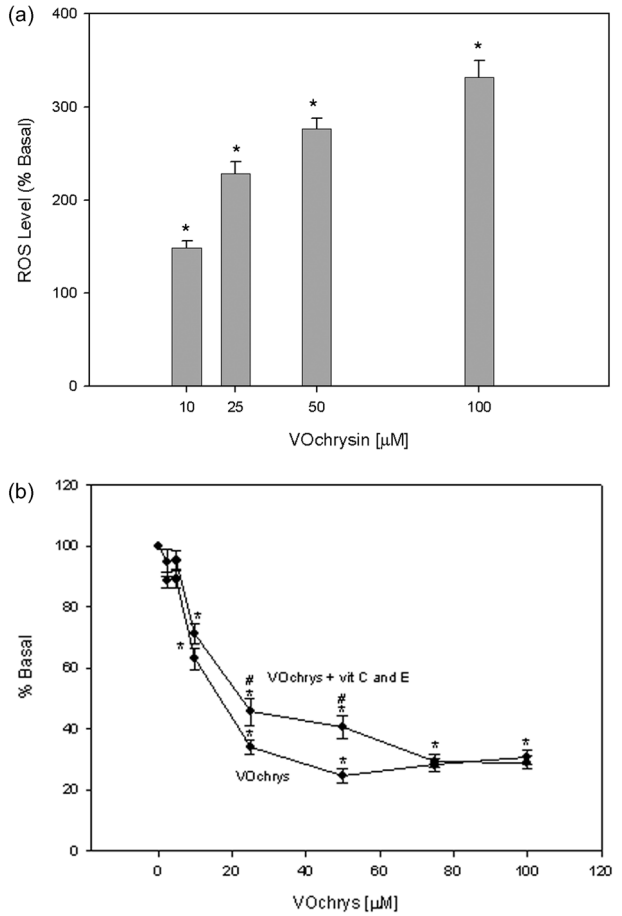

Fig. 5 (a) Induction of ROS by VOchrys in MG-63 cell line. Cells were incubated with growing concentrations of VOchrys at 37 °C for 24 h. ROS production in the cells was evaluated through the oxidation of DHR-123 to Rhodamine123. Results represent the mean ± SEM, n = 9, *significant differences vs. control $p < 0.01$. (b) Effects of VOchrys on MG-63 human osteosarcoma cell line proliferation in the presence of scavengers of ROS. Cells were incubated with different concentrations of the VOchrys at 37 °C for 24 h or with VOchrys in the presence of a mixture of ROS scavengers (vitamin C plus vitamin E, 50 μM each). Results are expressed as the percentage of the basal level and represent the mean ± SEM (n = 18). * significant difference in comparison with the basal level ($p < 0.01$) . # significant differences between treatments ($p < 0.05$).

recovering in cell survival was obtained at 25 and 50 μM. Nevertheless, at 75 and 100 μM the compound was more toxic and no recovery could be detected (Fig. 5B).

It is generally known that the GSH-related thiols participate in many important biological reactions, including the protection of cell membranes against oxidative damage. Vanadium toxicity has been previously associated with its capacity to induce the formation of ROS, probably by interacting with mitochondrial redox centers.[42]

To get a broader knowledge of the factors involved in the cellular redox status, the GSH/GSSG system was then investigated. GSH is one of the major reducing agents in mammalian cells. This thiol acts by sequestering free radicals and regulating the redox status by means of the couple GSH/GSSG.[43] A sustained increase in ROS levels may cause an accumulation of GSSG inside the cells. Because of this, the determination of GSH/GSSG ratio is relevant to the investigation of oxidative stress.[44]

As can be seen from Fig. 6, VOchrys induced a decrease in the GSH/GSSG ratio in the MG-63 human osteosarcoma cell line in relation to the concentration reaching a 40% value at the higher doses (50 and 100 μM). Taking into consideration the GSH level (Fig. 6), at these concentrations, there is a decrease to *ca.* 60% of the basal value in a non-concentration related manner. As a whole, these results indicate that the decrease of the GSH/GSSG ratio was due not only to a decrease in the level of GSH but also to an accumulation of GSSG.

On the other hand, Table 1 shows the basal levels of GSH in MG-63 and in two osteoblast cell lines of murine origin.

Overall, it can be assumed that the free radicals decrease the concentration of important cellular compounds and cause weakness of the antioxidant system, making the cells more vulnerable to oxidative damage.

Even though MG-63 cells have the highest level of the protective thiol, the deleterious effect of VOchrys was higher on this cell line as it has been described in the viability studies (see Fig. 1, ESI†). These results indicate that the sensitivity to the vanadium complex might depend on the cellular origin and indicate that the depletion of GSH concentration would mediate the cytotoxic action of VOchrys. Similar results have been previously reported for another vanadyl(IV) complex in osteoblast-like cells in culture.[24]

In some *in vivo* models it was also previously reported that vanadate, a vanadium(V) species, caused a further decrease of GSH levels in diabetic rat liver.[45] Besides, it has also been reported that the GSH levels are related to the neoplastic transformation and toxicity in mouse embryo cells.[46]

To confirm that the depletion of GSH played a role in the cytotoxic effect of the complex in MG-63 cells, a viability experiment was carried out in the presence of VOchrys and with the addition of 1 mM GSH (see Fig. 6). Results showed a statistically significant improvement of cell viability in comparison to the incubations without GSH addition.

Apoptosis study. Apoptosis is a physiological process of cell death and is enhanced in the presence of injuring agents. Apoptosis produces various modifications in the cell structure, mainly at the cellular membrane level. Apoptosis may be triggered by an extrinsic pathway mediated by receptors on the surface of the cells or can be produced by the endoplasmic reticulum or mitochondrial stress (intrinsic pathway). As a consequence, a genetic program that leads to cell death is activated. This process is accompanied by characteristic morphological changes in the nucleus and the cytoplasm. Because of this, apoptosis can be assessed by using several characteristic features of programmed cell death.

One of the first alterations that can be defined is the externalization of the phosphatidyl serine (PS), at the outer plasma membrane leaflet. Another feature of apoptosis is the alteration of the mitochondria membrane potential (MMP) that leads to the release of cytochrome *c*, the activation of the caspase pathway and finally to DNA fragmentation.

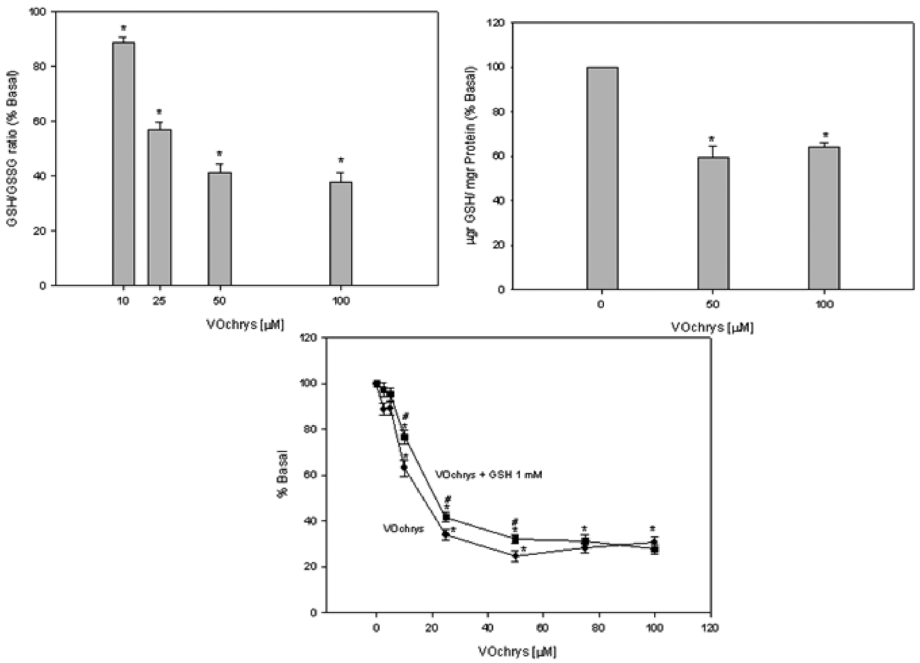

Fig. 6 Effect of VOchrys on GSH/GSSG ratio, GSH cellular levels and cell viability in the presence of GSH in MG-63 cells. Results are expressed as mean ± SEM of three independent experiments, * significant differences vs. basal ($p < 0.01$) # significant differences between treatments ($p < 0.05$).

Table 1 Basal levels of GSH in osteoblast cell lines

Cell line	GSH (μg GSH per mg protein)
MC3T3-E1	3.47 ± 0.26[a]
UMR106	6.15 ± 0.39[a]
MG-63	11.83 ± 0.80

[a] From ref. 32.

Independently of the cellular type and the nature of the trigger agent, the externalization of PS is always present in the earlier apoptotic events. Annexin V–FITC is a fluorescent probe with a high affinity for PS, allowing its determination by fluorescence assays. Our flow cytometry results showed an increment of the apoptotic cells in the presence of VOchrys in comparison to untreated cells (basal condition).

Table 1 (ESI†) displays the quantification of apoptotic cells determined by flow cytometry in MG-63 cells incubated with different concentrations of VOchrys. As can be seen, after 24 h of incubation, untreated MG-63 cells under basal conditions showed *ca.* 94% of living cells, defined as Annexin V (−) PI (−), 0.3% of Annexin V (+) PI (−) or early apoptotic cells and 3.3% of late apoptotic/necrotic cells (Annexin V (+) and PI (+)). These percentages vary with the complex concentration, increasing the amount of apoptotic and apoptotic/necrotic cells proportionally to the complex concentration.

These results demonstrated a strong deleterious action of VOchrys on the MG-63 cell line ($p < 0.01$).

For a better understanding of the early apoptotic events, new experiments at shorter times of incubation (3 h and 6 h) were performed. After 3 h of treatment, VOchrys did not cause any change in the externalization of PS either at 25 or at 100 μM (data not shown). Nevertheless, after 6 h of incubation, these concentrations of the complex produced *ca.* 20% of apoptotic cells (Annexin V+) with statistically significant differences under the basal condition but not between the two concentrations (see Fig. 7A and 7B). The amount of apoptotic cells was greater at 6 h than at 24 h since at the latter time, this fraction diminished to give rise to the apoptotic/necrotic population.

On the other hand, the results from our laboratory have shown that a complex of vanadium(v) with salicylaldehyde semicarbazone (V-Salsem) also caused an increase in the number of apoptotic cells as a function of its concentration.[47]

Moreover, reports from the literature on simple inorganic vanadium species such as orthovanadate induced also the generation of typical features of apoptosis including DNA fragmentation, loss of MMP, production of ROS and activation of caspase-3 in thyroid cancer cells.[48] Vanadium pentoxide, another vanadium(v) inorganic compound, has been shown to cause an increase of oxidative stress and apoptosis in human umbilical vein endothelial cells (HUVECs).[49]

Besides, it has been previously reported by Zhao *et al.* that $VOSO_4$, $NaVO_3$ and vanadyl complexes with organic ligands induced oxidative stress on mitochondria and thus caused an increase in different apoptotic mechanisms such as the opening of mitochondria membrane pores which led to collapse of MMP and cytochrome *c* release.[50]

A great deal of attention has been focused lately on the emerging role of mitochondria as regulators of cell life–cell death transition, in both necrotic and apoptotic forms of cell death.[51] The normal performance and survival of cells that

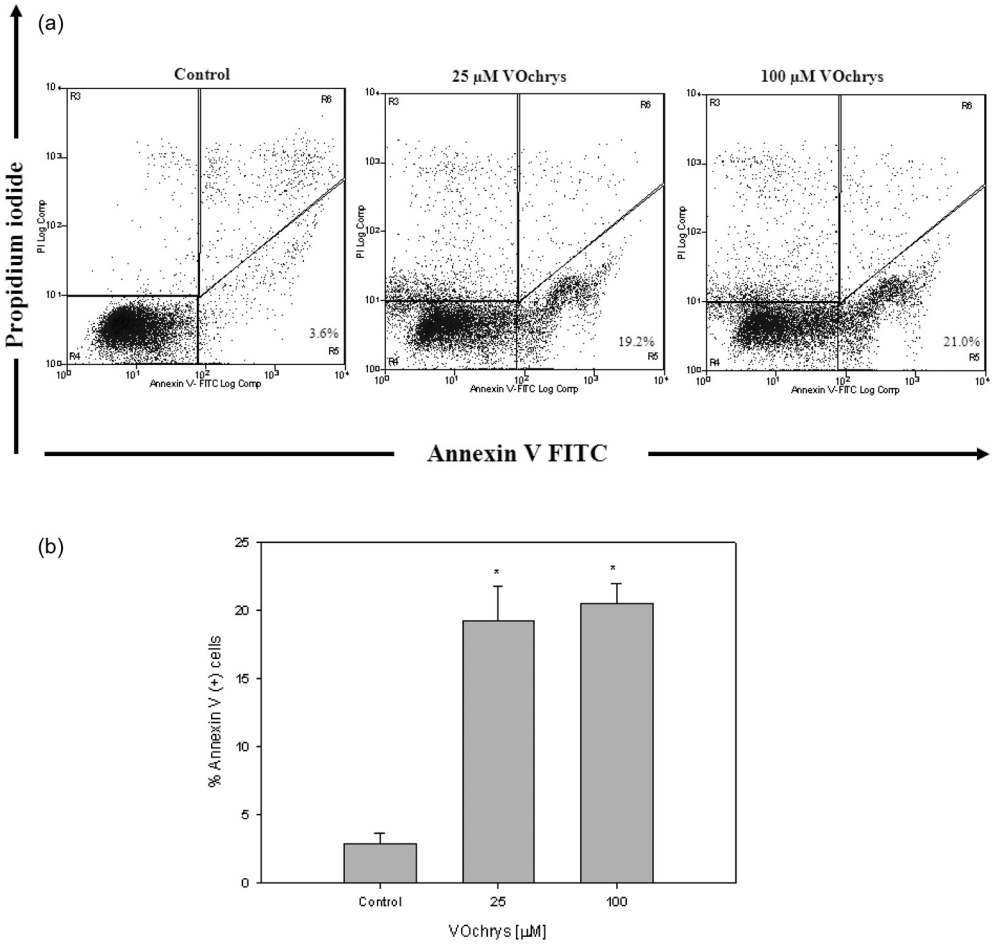

Fig. 7 (a) Effect of VOchrys on apoptosis assessed by flow cytometry using Annexin V-FITC/PI staining. MG-63 cells were treated with 0 (control), 25 and 100 μM of the complex at 37 °C during 6 h. Plots are representative of three independent experiments. Numbers in R5 quadrant indicate the Annexin V (+) cells. (b) Graphical bars show the percentage of Annexin V (+) cells. Results are expressed as the mean ± SEM, $n = 9$, * significant differences vs. control ($p < 0.01$).

Table 2 MMP disruption by VOchrys in MG-63 cells

VOchrys [μM]	Disrupted MMP cells	Disrupted MMP cells in the presence of 1 mM GSH
0	13%	
10	16%	13%[a]
25	30%	ND
100	52%	43%[a]

[a] Significant difference ($p < 0.01$) between treatments at the same concentration of VOchrys. ND, not determined.

have high energy requirements depend on the maintenance of the MMP. Measurement of MMP is therefore essential for extending the understanding of molecular mechanisms.

Rhodamine 123 is a lipophilic and low toxicity compound with high affinity for mitochondria and it is broadly used for the measurement of MMP.[52,53]

In our model system, VOchrys caused a dissipation of MMP in a dose dependent trend (see Table 2). Under control conditions there were 13% of apoptotic cells. This fraction increased with the concentration of the complex.

In order to clarify the role of oxidative stress in the alteration of MMP, this parameter was evaluated in the presence of different concentrations of VOchrys with a previous incubation of the cells with 1 mM of GSH. As observed in Table 2, the apoptotic cell population significantly diminished, confirming that the MMP alteration was produced by oxidative stress.

These results correlate with a previous report on the effect of a vanadium(IV) compound with oxodiacetate on MMP in two murine osteoblast-like cells in culture.[24] Huang *et al.* have also shown that vanadate induced the loss of MMP through the generation of H_2O_2 in mouse epidermal cells, promoting the death by apoptosis.[54] Similar results were reported by D'Cruz *et al.* for vanadocenes in germinal cells.[55]

Moreover, one of the most important pathways of apoptosis is the activation of caspase cascade. Caspases (cysteine-requiring aspartate protease) are a family of proteases that mediate cell death and are important for the process of apoptosis. Caspase 3 is one of the critical members of this family.[56–58] It plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as cell blebbing.[59]

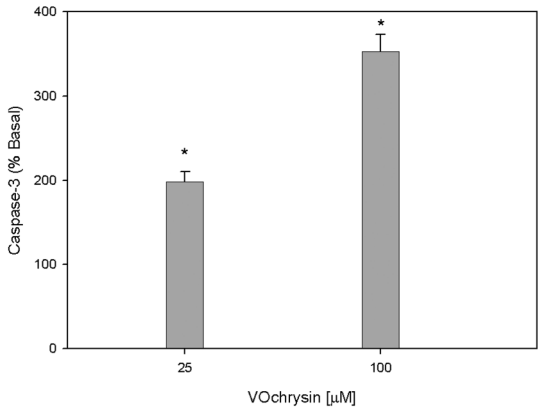

Fig. 8 Effect of VOchrys on caspase 3 activation. Enzymatic activity of caspase 3 was determined with the caspase 3 specific colorimetric substrate Ac-DEVD-pNA. MG-63 cells were treated with 0 (control), 25 and 100 μM of the complex at 37 °C during 6 h and then harvested, lysed and analyzed. Results are expressed as mean ± SEM of three independent experiments, $n = 12$, * significant differences vs. control ($p < 0.01$).

From Fig. 8 it can be seen that caspase 3 is activated as a function of the complex concentration ($p < 0.01$), demonstrating that the apoptotic action of the complex is in accordance with the Annexin V and MMP results. Besides, at the shortest time used in the present study (6 h), the activation of caspase 3 was better than the assay with Annexin V to detect early apoptosis since a significant effect with the concentration of the complex could be determined for caspase 3 and not for the Annexin V measurements. Nevertheless, in the two tests the differences between untreated and treated conditions were always statistically significant.

Collectively, the antiproliferative concentrations of the drug induce typical features of apoptosis including PS externalization, loss of MMP, production of ROS and activation of caspase-3.

Recent papers have shown that in isolated rat liver hepatocytes as well as in a N27 dopaminergic neuronal cell model, vanadium induced cytotoxicity and an increase in the levels of caspase 3.[60,61]

Cell cycle analysis and DNA fragmentation. The entry of cells into each phase of the cell-cycle is carefully regulated by different checkpoints. One theme emerging from drug discovery is to develop agents that target the cell-cycle checkpoints.[62]

Apoptotic cells have deficient DNA content, and when stained with a DNA-specific fluorochrome, they can be recognized by flow cytometry as cells with less DNA than G1 cells. On the DNA content frequency histograms, they form a characteristic "sub-G1" peak.[63–65]

The degree of DNA degradation varies depending on the stage of apoptosis, cell type, and often the nature of the apoptosis-inducing agent. It is possible to use flow cytometry to study DNA population species from apoptotic cells.

The alteration of the cell cycle in control and VOchrys treated cells was analyzed by flow cytometry as a function of the incubation time and the complex concentration. As shown in Fig. 9A and 9C, 25 μM VOchrys arrested the cell cycle in G2 phase at 6 h of incubation ($p < 0.05$). After this time the cells were conveyed to apoptosis (evaluated by sub-G1 peak).

Treated cells undergoing apoptosis after 6, 16 and 24 h treatments showed a significant increase in the sub-G1 peak ($p < 0.01$) (Fig. 9B and 9C). Besides, a clear reduction of cells in the G2/M phase and an increase in the sub-G1 peak could be determined at 25 and 100 μM of VOchrys compared to untreated cells. Moreover, the sub-G1 peak at 100 μM was greater than that at 25 μM. These results are in agreement with those of caspase 3 activation and with the disruption of the MMP, confirming those findings.

Altogether, the deleterious effects of VOchrys that convey the tumor cells to death are dependent on the complex concentration.

Conclusions

As part of a project devoted to research on metal complexes of ligands with interesting pharmacological or biological properties, in this work we focused our attention on the antitumor effects of VOchrys, an oxovanadium(IV) complex with the flavonoid chrysin.

Since osteosarcoma is the most frequent bone tumor, the human osteosarcoma cell line MG-63 was chosen for this study.

A comprehensive study has been carried out for the first time on the promissory antitumor properties of this complex in a human osteosarcoma cell line. We have demonstrated that the complexation of the flavonoid chrysin with VO(IV) improved the antitumor activity of the ligand and the comparison of the antiproliferative effects in other tumor and normal bone related cells showed the most potent effect of the complex in the human osteosarcoma-derived cell line.

VOchrys caused cyto- and genotoxicity in a concentration dependent manner. It interacted with lysosomes and mitochondria as well as with the cellular DNA.

The main mechanisms of action involved in the antitumor effects of VOchrys were mediated by an increase in the ROS level which triggered the apoptosis events as determined by the externalization of PS, DNA fragmentation and caspase-3 activation.

This research intends to contribute to the knowledge of the biochemical properties, as well as the mechanisms of the cellular and molecular antitumor effects of vanadium compounds.

Since the antiproliferative action of the complex was more pronounced in the human osteosarcoma derived cell line, it would be interesting to test this complex in further *in vivo* studies for cancer treatments.

Experimental

Materials

Tissue culture materials were purchased from Corning (Princeton, NJ, USA), Dulbecco's Modified Eagle's Medium (DMEM),

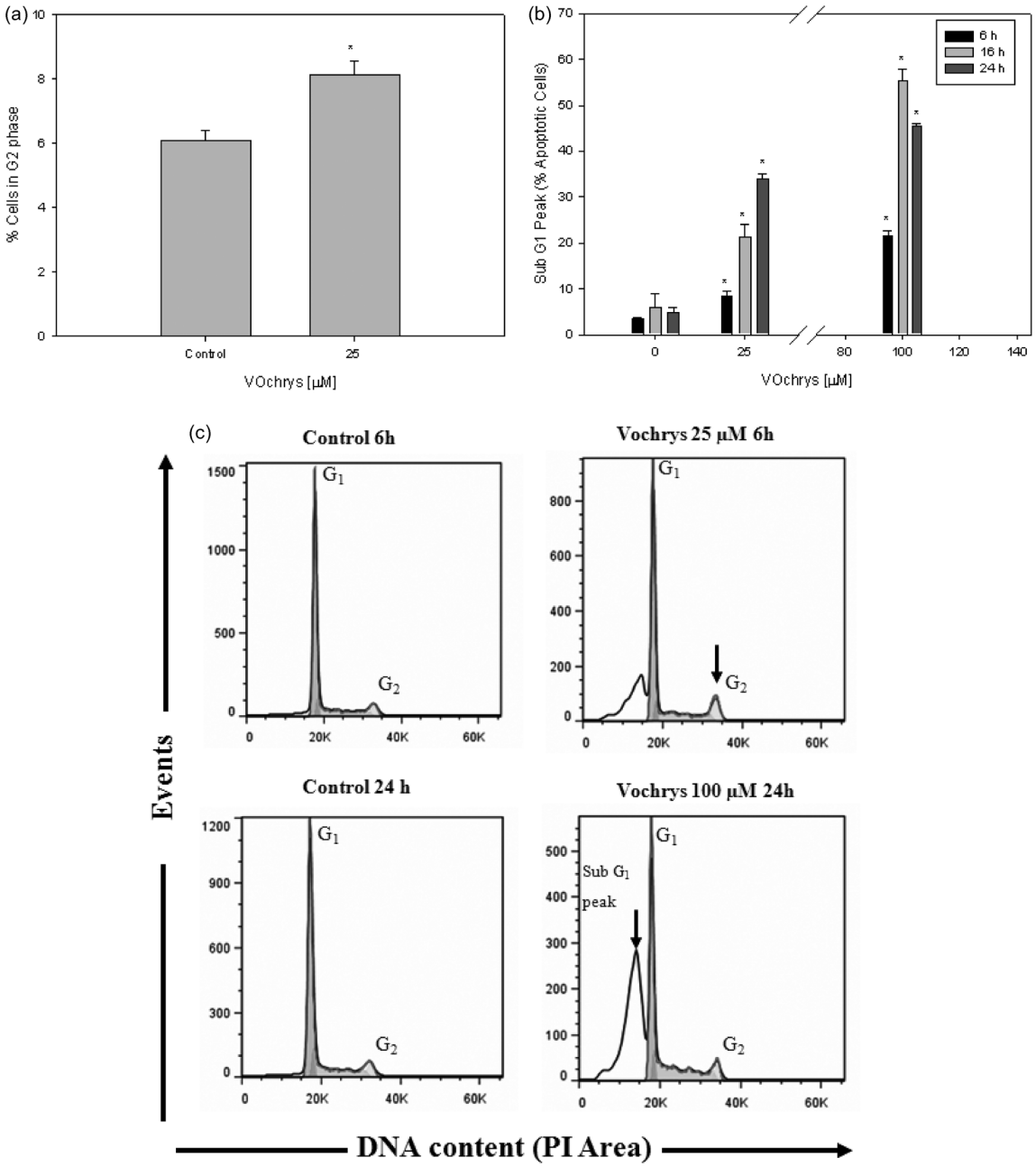

Fig. 9 Effect of VOchys on Cell Cycle Arrest and DNA fragmentation. (a) Effects of VOchrys on Cell Cycle Arrest (phase G2). MG-63 cells were treated with 0 (control) and 25 μM of VOchrys at 37 °C during 6 h. Graphical bars show the percentage of G2 phase cells. Results are expressed as the mean ± SEM, $n = 9$, * significant differences vs. control ($p < 0.05$). (b) MG-63 cells were treated with 0 (control), 25 and 100 μM of the complex at 37 °C during 6, 16 and 24 h. Graphical bars show the percentage of Sub G1 peak cells. Results are expressed as the mean ± SEM, $n = 9$, * significant differences vs. control ($p < 0.01$). (c) Effect of VOchrys on Cell Cycle and DNA fragmentation assessed by flow cytometry using PI stain. Plots are representative of three independent experiments.

TrypLE™ from Gibco (Gaithersburg, MD, USA), and fetal bovine serum (FBS) from Internegocios SA (Argentina). Dihydrorhodamine (DHR) and rhodamine 123 were purchased from Molecular Probes (Eugene, OR). Annexin V and fluorescein isothiocyanate (FITC)/PI were from Invitrogen Corporation (Buenos Aires, Argentina). Cytochalasin B from *Dreschslera dematioidea* was purchased from Sigma Chemical Co (St. Louis, MO, USA), Bleomycin (BLM) was kindly provided by Gador S.A Buenos Aires, Argentina. SyberGreen and low melting point agarose were purchased from Invitrogen Corporation (Buenos Aires, Argentina). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Fresh stock solutions of the complex and the free ligand were prepared in dimethyl sulfoxide (DMSO) at 20 mM concentration and diluted in DMEM according to the concentrations

indicated in the legends of the figures. Precautions should be taken with the maximum concentration of DMSO in the well plate. We used 0.5% as the maximum DMSO concentration in order to avoid the toxic effects of this solvent on the osteoblasts.

Methods

Synthesis of VOchrys. VOchrys was synthesized according to previously reported results.[1] Briefly, chrysin (0.5 mmol) was mixed with vanadyl acetylacetonate (0.25 mmol) in absolute ethanol and refluxed for *ca.* 1.30 h (final pH = 5). The hot green suspension was filtered, washed three times with absolute ethanol, and air-dried. Anal. Calcd for $C_{64}H_{48}O_{20}V_2$: C, 62.0; H, 3.9; V, 8.2. Exp.: C, 62.0; H, 4.1; V, 8.1. Yield: 70%. The identification of the complex was done by using FTIR.

Preparation of VOchrys solutions. Fresh stock solutions of the complex and the free ligand were prepared in DMSO at 20 mM and diluted according to the concentrations indicated in the legends of the figures.

Cell culture and incubations. The MG-63 cell line was purchased from ATCC (CRL1427™), and MG-63 human osteosarcoma cells were grown in DMEM containing 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a 5% CO_2 atmosphere. Cells were seeded in a 75 cm^2 flask and when 70–80% of confluence was reached, cells were subcultured using 1 mL of TrypLE™ per 25 cm^2 flask. For experiments, cells were grown in multi-well plates. When cells reached the desired confluence, the monolayers were washed with DMEM and were incubated under different conditions according to the experiments.

Cell viability: crystal violet assay. A mitogenic bioassay was carried out as described by Okajima *et al.*[66] with some modifications. Briefly, cells were grown in 48 well plates. For experiments, the cells (3×10^4 cells per mL) were grown for 24 h at 37 °C. Then, the monolayer was incubated with different concentrations (2.5–100 μM) of oxovanadium(IV) or the free ligand or with different concentrations of the complex for the proliferation assay. Besides, incubations were also carried out with the complex plus reduced glutathione (GSH) and with a mixture of vitamins C and E. Cells were preincubated with GSH (1 mM) during 2 h. Then the thiol was eliminated before the addition of the complex. For other experiments the cells were incubated with different concentrations of the complex plus a mixture of vitamins C and E (50 μM each) during 24 h.[25]

After this treatment, the monolayers were washed with PBS and fixed with 5% glutaraldehyde–PBS at room temperature for 10 min. After that, they were stained with 0.5% crystal violet/25% methanol for 10 min. Then, the dye solution was discarded and the plate was washed with water and dried. The dye taken up by the cells was extracted using 0.5 mL per well 0.1 M glycine/HCl buffer, pH 3.0/30% methanol and transferred to test tubes. Absorbance was read at 540 nm after a convenient sample dilution. It was previously shown that under these conditions, the colorimetric bioassay strongly correlated with cell proliferation measured by cell counting in a Neubauer chamber.[67]

Cell morphology. Cells were grown on glass coverslips and incubated under control conditions (without complex addition) or with different concentrations of VOchrys in serum-free DMEM at 37 °C for 24 h. Then, the cells were fixed and stained with Giemsa.[36] Samples were observed under light microscopy.

Neutral red assay. The neutral red accumulation assay was performed according to Borenfreund and co-workers.[27] Cells were plated in 96 well culture plates (2.5 × 104 cells per well). Cells were treated with different VOchrys concentrations for 24 h at 37 °C in 5% CO_2 in air. After treatment, the medium was replaced by one containing 100 μg mL^{-1} neutral red (NR) dye and cells were incubated for another 3 h. Then, neutral red medium was discarded, the cells were rinsed twice with warm (37 °C) PBS (pH 7.4) to remove the non-incorporated dye, and 100 μL of a 50% ethanol and 1% acetic acid solution was added to each well to fix the cells releasing the neutral red into solution. The plates were shaken for 10 min, and the absorbance of the solution in each well was measured in a microplate reader (7530, Cambridge Technology, Inc., USA) at 540 nm, and compared with wells with untreated cells. Optical density was plotted as a percentage of the control.

MTT assay. The MTT assay was based on a report previously described by Mosmann.[28] Briefly, cells were seeded in a 96-multiwell dish, allowed to attach for 24 h and treated with different concentrations of VOchrys at 37 °C for 24 h. After this treatment, the medium was changed and the cells were incubated with 0.5 mg mL^{-1} MTT under normal culture conditions for 3 h. Cell viability was marked by the conversion of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) to a colored formazan by mitochondrial dehydrogenases. Color development was measured spectrophotometrically in a Microplate Reader (7530, Cambridge technology, Inc., USA) at 570 nm after cell lysis in DMSO (100 μL per well). Cell viability is shown graphically as a percentage of the control value.

Cytokinesis-block micronucleus (MN) assay. The cytokinesis-block micronucleus technique was set up with cultures in the log phase of growth. Cells were seeded onto pre-cleaned coverslips placed in 35 mm Petri dishes at a density of 3×10^4 cells per dish and incubated at 37 °C for 24 h. Then, the cells were treated with different concentrations of the complex with cytochalasin B (4.5 μg mL^{-1}). After 24 h, the cells were rinsed and subjected to hypotonic conditions with 0.075% KCl at 37 °C for 5 min, fixed with pure methanol at −20 °C for 10 min and stained with a 5% Giemsa solution. After staining, the coverslips were air-dried and placed down onto pre-cleaned slides using Depex mounting medium. For the MN assay, 1000 binucleated (BN) cells were scored at 400× magnification per experimental point from each experiment. The examination criteria employed were reported by Fenech.[68] Briefly, the criteria employed in identifying micronuclei (MNi) were: diameter smaller than 1/3 of that of the main nuclei, non-refractibility, the same staining intensity as or lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei and MNi

boundary distinguishable from main nuclei boundary.[69] As the positive control, cells were exposed to a pulse of 30 minutes of 1 μg mL^{-1} bleomycin 24 hours before the harvest.

Single cell gel electrophoresis (SCGE) assay. For the detection of DNA strand breaks the single cell gel electrophoresis ('comet') assay was used in the alkaline version, based on the method of Singh *et al.*[70] with minor modifications. Under alkaline conditions, DNA loops containing breaks lose supercoiling, unwind and are released from the nuclei and form a 'comet-tail' by gel electrophoresis. For this experiment, 2×10^4 cells were seeded in a twelve-well plate; 24 h later the cells were incubated with various concentrations of the complexes. After treatment, cells were suspended in 0.5% low melting point agarose and immediately poured onto glass microscope slides. Slides were immersed in an ice-cold prepared lysis solution at darkness for 1 h (4 °C) in order to lyse the cells, remove cellular proteins and to permit DNA unfolding. Immediately after this, slides were put in a horizontal electrophoresis tank containing 1 mM Na_2EDTA, 0.3 M NaOH (pH 12.7) and then electrophoresis was performed for 30 min at 25 V (4 °C). Afterwards, slides were neutralized and stained with Syber Green. Analysis of the slides was performed in an Olympus BX50 fluorescence microscope. Cellular images were acquired with a Leica IM50 Image Manager (Imagic Bildverarbeitung AG). A total of 50 randomly captured cells per experimental point of each experiment was used to determine the tail moment (the product of tail length by tail DNA percentage) using a free comet scoring software (Comet Score version 1.5). Two parallel slides were performed for each experimental point. Independent experiments were repeated twice. A pulse of 20 minutes of 10 μg mL^{-1} bleomycin just before the cells were harvested was employed as the positive control.

Determination of reactive oxygen species (ROS) production. Oxidative stress in the osteoblasts was evaluated by measurement of intracellular production of reactive oxygen species (ROS) after incubation of the cell monolayers with different concentrations of the complex during 4 h at 37 °C. ROS generation was determined by oxidation of DHR-123 to rhodamine by spectrofluorescence as we have previously described.[36]

Fluorometric determination of cellular GSH and GSSG levels. GSH and GSSG levels were determined in osteoblasts in culture as follows. Confluent osteoblast monolayers from 24 well dishes were incubated with different concentrations of VOchrys at 37 °C for 24 h. Then, the monolayers were washed with PBS and harvested by incubating them with 250 μL Triton 0.1% for 30 min. For GSH determination, 100 μL aliquots were mixed with 1.8 mL of ice cold phosphate buffer (Na_2HPO_4 0.1 M–EDTA 0.005 M, pH 8) and 100 μL *o*-phthaldialdehyde (0.1% in methanol) as it was described by Hissin and Hilf.[71] For the determination of GSSG, 100 μL aliquots were mixed with 1.8 mL NaOH 0.1 M and *o*-phthaldialdehyde as before. Previously, to avoid GSH oxidation the cellular extracts for GSSG determination were incubated with 0.04 M of *N*-ethylmaleimide (NEM). The fluorescence at an emission wavelength of 420 nm was determined after excitation at 350 nm. Standard curves with different concentrations of GSH were processed in parallel. The protein content in each cellular extract was quantified using the Bradford assay.[72]

The ratio GSH/GSSG, which is a better marker for the cellular redox status, was calculated as % basal for all the experimental conditions.

Measurement of the exposure of phosphatidyl serine (PS) by Annexin V–FITC/PI staining. Cells in the early and late stages of apoptosis were detected with Annexin V–FITC and propidium iodide (PI) staining. Cells were treated with 25 and 100 μM VOchrys and incubated for 3, 6 and 24 h prior to analysis. For the staining, cells were washed with PBS and adjusted to a concentration of 1×10^6 cell per mL in 1× binding buffer. To 100 μL of cell suspension, 2.5 μL of Annexin V–FITC and 2 μL PI (250 μg mL^{-1}) were added and incubated for 15 min at room temperature prior to analysis. Cells were analyzed using a CyAn™ ADP flow cytometer (Beckman Coulter, USA) and Summit v4.3 software. For each analysis, 10 000 counts, gated on a FSC *vs.* SSC dot plot, were recorded. Four subpopulations were defined in the dot plot: the undamaged vital (Annexin V−/PI−), the vital mechanically damaged (Annexin V−/PI+), the apoptotic (Annexin V+/PI−), and the secondary necrotic (Annexin V+/PI+) subpopulations.

Caspase 3 assay. Determination of caspase 3, one of the main effector caspases, was carried out with a commercial kit (Caspase 3 Assay Kit Colorimetric, SIGMA) following the recommendations of the manufacturers.

Briefly, cells were grown in a 25 cm^2 flask at 37 °C for 24 h (2.5×10^6 cells). Then, the monolayer was incubated with different concentrations (25 and 100 μM) of the complex. After this treatment, the monolayers were washed with PBS and were harvested with TrypLE at 37 °C for 5 min.

After that, lysis buffer 1× was added and incubated in an ice-cold bath for 15 min. Then, the tubes were centrifuged at 17 000 rpm for 15 min and the supernatants were collected. After that, the reaction mixtures were prepared according to the manufacturers' indications and the absorbance was read at 420 nm.

Measurement of cell cycle/DNA content. DNA content in G1/G0, S, and G2/M phases was analyzed using flow cytometry.[64,65]

Cells were seeded on 6 well plates, cultured during 24 h and then treated with 25 μM and 100 μM of VOchrys for 6, 16 and 24 h. The harvested cells were washed with PBS, fixed and permeabilized with 70% ice-cold ethanol for more than 2 h. Subsequently, cells were resuspended in freshly staining buffer (15 mg mL^{-1} of PI and 15 mg mL^{-1} DNase-free RNase prepared in PBS containing 2 mM EDTA) and incubated for 15 min at 37 °C. After gating out cellular aggregates, the cell cycle distribution analysis was performed on CyAn™ ADP flow cytometer using Summit v4.3 software for the acquisition. For each sample, at least 10 000 cells were counted and plotted on a single parameter histogram. The percentage of cells in the G1/G0, S, G2/M phases and sub-G1 peak was then calculated using FlowJo 7.6 software (Watson model).

Statistical analysis. At least three independent experiments were performed for each experimental condition in all the

biological assays. The results are expressed as the mean ± standard error of the mean (SEM). Statistical differences were analyzed using the analysis of variance method (ANOVA) followed by the test of least significant difference (Fisher).

Acknowledgements

This work was partly supported by UNLP (11X/554), CONICET (PIP 1125 and 0938), and ANPCyT (PICT 2008-2218 and PICT-2010-0981) from Argentina. ALDV, CIM and SBE are members of the Carrera del Investigador, CONICET, Argentina. IEL has a fellowship from ANPCyT, Argentina. VP and MBF are members of the Sistema Nacional de Investigadores (SNI) of the Agencia Nacional de Investigación e Innovación (ANII) from Uruguay. LGN is a fellowship from CONICET, Argentina and PAMW is member of the Carrera del Investigador CICPBA. Besides, the authors would like to thank Inés Tiscornia for management of the cells and Prof. María del Carmen Bernal for her careful revision of the manuscript.

References

1 L. G. Naso, E. G. Ferrer, L. Lezama, T. Rojo, S. B. Etcheverry and P. A. Williams, *J. Biol. Inorg. Chem.*, 2010, **15**, 889–902.
2 A. D. McNaught and A. Wilkinson, IUPAC "Flavonoids (isoflavonoids and neoflavonoids)", *IUPAC Compendium of Chemical Terminology*, Blackwell Scientific, Oxford, 2nd edn, 1997.
3 P. G. Pietta, *J. Nat. Prod.*, 2000, **63**, 1035–1042.
4 N. Sugihara, A. Takayuki, M. Ohnishi and K. Furuno, *Free Radicals Biol. Med.*, 1999, **27**, 1313–1323.
5 X. Zheng, W. D. Meng, Y.-Y. Xu, J. G. Cao and F. L. Qinga, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 881–884.
6 C. A. Rice-Evans, in *Flavonoids in health and disease*, ed. L. Packer, Marcel Dekker, New York, 2nd edn, 2003.
7 R. Kohen and A. Nyska, *Toxicol. Pathol.*, 2002, **30**, 620–650.
8 I. Rahman, S. K. Biswas and P. A. Kirkham, Regulation of inflammation and redox signaling by dietary polyphenols, *Biochem. Pharmacol.*, 2006, **72**(11), 1439–1452.
9 M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur and J. Telser, *Int. J. Biochem. Cell Biol*, 2007, **39**, 44–84.
10 V. Kuntić, I. Filipović and Z. Vujić, *Molecules*, 2011, **16**, 1378–1388.
11 A. N. Koc, S. Silici, D. Ayangil, A. Ferahbas and S. Cankay, *Mycoses*, 2005, **48**, 205–210.
12 M. Cardenas, M. Marder, V. C. Blank and L. P. Roguin, *Bioorg. Med. Chem.*, 2006, **14**, 2966–2971.
13 X. M. Yu, T. Phan, P. N. Patel, R. Jaskula-Sztul and H. Chen, *Cancer*, 2013, **119**, 774–781.
14 X. Li, J. N. Wang, J. M. Huang, X. K. Xiong, M. F. Chen, C. N. Ong, H. M. Shen and X. F. Yang, *Toxicol. in Vitro*, 2011, **25**, 630–635.
15 F. H. Nielsen, in *Metal Ions in Biological Systems, Vanadium and Its Role in Life*, ed. H. Sigel and A. Sigel, Marcel Dekker, New York, 1995, vol. 31, pp. 543–574.
16 C. Slebodnicj, B. J. Hamstra and V. L. Pecoraro, *Struct. Bonding*, 1997, **89**, 51–107.
17 K. Srivastava and M. Z. Mehdi, *Diabet. Med.*, 2004, **22**, 2–13.
18 D. A. Barrio, E. R. Cattaneo, M. C. Apezteguia and S. B. Etcheverry, *Can. J. Physiol. Pharmacol.*, 2006, **84**, 765–775.
19 A. M. Evangelou, *Crit. Rev. Oncol. Hematol.*, 2002, **42**, 249–265.
20 M. S. Bhuiyan and K. Fukunaga, *J. Pharmacol. Sci.*, 2009, **110**, 1–13.
21 N. Shioda, F. Han and K. Fukunaga, *Int. Rev. Neurobiol.*, 2009, **85**, 375–387.
22 C. Djordjevic, in *Metal Ions in Biological Systems, Vanadium and Its Role in Life*, ed. H. Sigel and A. Sigel, Marcel Dekker, New York, 1995, vol. 31, pp. 595–616.
23 S. B. Etcheverry and P. A. M. Williams, New developments in medicinal chemistry, in *Medicinal Chemistry of Copper and Vanadium Bioactive Compounds*, ed. M. P. Ortega and I. C. Gil, Nova Science, Hauppauge, 2009, pp. 105–129.
24 J. Rivadeneira, A. N. Di Virgilio, D. A. Barrio, C. I. Muglia, L. Bruzzone and S. B. Etcheverry, *Med. Chem.*, 2010, **6**, 9–23.
25 S. B. Etcheverry, E. G. Ferrer, L. G. Naso, J. Rivadeneira, V. Salinas and P. A. M. Williams, *J. Biol. Inorg. Chem.*, 2008, **13**, 435–447.
26 A. L. Di Virgilio, J. Rivadeneira, C. I. Muglia, M. A. Reigosa, N. Butenko, I. Cavaco and S. B. Etcheverry, *Biometals*, 2011, **24**, 1153–1168.
27 E. Borenfreund and J. A. Puerner, *J. Tissue Cult. Methods*, 1984, **9**, 7–9.
28 T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55–63.
29 H. Stopper and S. O. Muller, *Toxicol. in Vitro*, 1997, **11**, 661–667.
30 H. Suzuki, K. Nagai, H. Yamaki, N. Tanaka and H. Umezawa, *J. Antibiot.*, 1969, **22**, 446–448.
31 A. R. Collins, V. L. Dobson, M. Dusinska, G. Kennedy and R. Stetina, *Mutat. Res.*, 1997, **375**, 183–193.
32 W. Liao, M. A. Nutt and M. G. Zhu, *Methods*, 2009, **48**, 46–53.
33 J. J. Rodríguez-Mercado, R. A. Mateos-Nava and M. A. Altamirano-Lozano, *Toxicol. in Vitro*, 2011, 25, 1996–2002.
34 I. E. León, A. L. Di Virgilio, D. A. Barrio, G. Arrambide, D. Gambino and S. B. Etcheverry, *Metallomics*, 2012, **4**, 1287–1296.
35 P. Villani, E. Cordelli, P. Leopardi, E. Siniscalchi, E. Veschetti, A. M. Fresegna and R. Crebelli, *Toxicol. Lett.*, 2007, **170**, 11–18.
36 A. M. Cortizo, L. Bruzzone, M. S. Molinuevo and S. B. Etcheverry, *Toxicology*, 2000, **147**, 89–99.
37 J. Ye, M. Ding, S. S. Leonard, V. A. Robinson, L. Millecchia, X. Zhang, V. Castranova, V. Vallyathan and X. Shi, *Mol. Cell. Biochem.*, 1999, **202**, 9–17.

38 Z. Zhang, C. Huang, J. Li, S. S. Leonard, R. Lanciotti, L. Butterworth and X. Shi, *Arch. Biochem. Biophys.*, 2001, **392**, 311–320.

39 M. A. M. Capella, Y. Qin, M. Lu and X. Gong, *Cell Biol. Int.*, 2008, **32**, 224–228.

40 C. C. Wang, Y. M. Chiang, S. C. Sung, Y. L. Hsu, J. K. Chang and P. L. Kuo, *Cancer Lett.*, 2008, **259**, 82–98.

41 H. K. Baumgartner, J. V. Gerasimenko, C. Thorne, L. H. Ashurst, S. L. Barrow, M. A. Chvanov, S. Gillies, D. N. Criddle, A. V. Tepikin, O. H. Petersen, R. Sutton, A. J. M. Watson and O. V. Gerasimenko, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2007, **293**, 296–307.

42 M. Valko, H. Morris and M. T. Cronin, *Curr. Med. Chem.*, 2005, **12**, 1161–1208.

43 D. P. Jones, J. L. Carlson, V. C. Mody, J. Y. Cai, M. J. Lynn and P. Sternberg, *Free Radicals Biol. Med.*, 2000, **28**, 625–635.

44 C. Hwang, A. J. Sinskey and H. F. Lodish, *Science*, 1992, **57**, 1496–1502.

45 A. K. Saxena, P. Srivastava, R. K. Kale and N. Z. Baquer, *Biochem. Pharmacol.*, 1993, **45**, 539–542.

46 E. Sabbioni, G. Pozzi, S. Devos, A. Pintar, L. Casella and M. Fischbach, *Carcinogenesis*, 1993, **14**, 2565–2568.

47 J. Rivadeneira, D. A. Barrio, G. Arrambide, D. Gambino, L. Bruzzone and S. B. Etcheverry, *J. Inorg. Biochem.*, 2009, **103**, 633–642.

48 A. P. Gonçalves, A. Videira, P. Soares and V. Máximo, *Life Sci.*, 2011, **12**, 11–12.

49 A. Montiel-Dávalos, A. Gonzalez-Villava, V. Rodriguez-Lara, L. F. Montaño, T. I. Fortoul and R. López-Marure, *J. Appl. Toxicol.*, 2012, **32**, 26–33.

50 Y. Zhao, L. Ye, H. Liu, Q. Xia, Y. Zhang, X. Yang and K. Wang, *J. Inorg. Biochem.*, 2010, **104**, 371–378.

51 E. J. Griffiths, *Cardiovasc. Res.*, 2000, **46**, 24–27.

52 R. Kim, K. Tanabe, Y. Uchida, M. Emi, H. Inoue and T. Toge, *Cancer Chemother. Pharmacol.*, 2002, **50**, 343–352.

53 Z. Darzynkiewicz, F. Traganos, L. Staiano-Coico, J. Kapuscinski and M. R. Melamed, *Cancer Res.*, 1982, **42**, 799–806.

54 C. Huang, Z. Zhang, M. Ding, J. Li, J. Ye, S. S. Leonard, H. M. Shen, L. Butterworth, Y. Lu, M. Costa, Y. Rojanasakul, V. Castranova, V. Vallyathan and X. Shi, *J. Biol. Chem.*, 2000, **275**, 32516–32522.

55 O. J. D'Cruz, Y. Donga and F. M. Uckun, *Biol. Reprod.*, 1999, **60**, 435–444.

56 D. W. Nicholson, A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle and Y. A. Lazebnik, *Nature*, 1995, **376**, 37–43.

57 H. Sakahira, M. Enari and S. Nagata, *Nature*, 1998, **391**, 96–99.

58 S. Kamada, H. Kusano, H. Fujita, M. Ohtsu, R. C. Koya, N. Kuzumaki and Y. Tsujimoto, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 8532–8537.

59 A. G. Porter and R. U. Janicke, *Cell Death Differ.*, 1999, **6**, 99–104.

60 M. J. Hosseini, N. Seyedrazi, J. Shahraki and J. Pourahmad, *Adv. Biosci. Biotechnol.*, 2012, **3**, 1096–1103.

61 H. Afeseh Ngwa, A. Kanthasamy, V. Anantharam, C. Song, T. Witte, R. Houk and A. G. Kanthasamy, *Toxicol. Appl. Pharmacol.*, 2009, **240**, 273–285.

62 C. J. Sherr, *Cancer Res.*, 2000, **60**, 3698–3695.

63 S. R. Umansky, B. A. Korol and P. A. Nelipovich, *Biochim. Biophys. Acta*, 1981, **655**, 9–17.

64 I. Nicoletti, G. Migliorati, M. C. Pagliacci, F. Grignani and C. Riccardi, *J. Immunol. Methods*, 1991, **139**, 271–280.

65 J. Gong, F. Traganos and Z. Darzynkiewicz, *Anal. Biochem.*, 1994, **218**, 314–319.

66 T. Okajima, K. Nakamura, H. Zhang, N. Ling, T. Tanabe, T. Yasuda and R. G. Rosenfeld, *Endocrinology*, 1992, **130**, 2201–2212.

67 A. M. Cortizo and S. B. Etcheverry, *Mol. Cell. Biochem.*, 1995, **145**, 97–102.

68 M. Fenech, *Mutat. Res.*, 2000, **455**, 81–95.

69 M. Fenech, *Mutat. Res.*, 1993, **1**, 35–44.

70 N. P. Singh, M. T. McCoy, R. R. Tice and E. L. Schneider, *Exp. Cell Res.*, 1988, **175**, 184–191.

71 P. J. Hissin and R. Hilf, *Anal. Biochem.*, 1976, **74**, 214–226.

72 M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.