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Original Articles

Oxovanadium-based inhibitors can drive redox-sensitive cytotoxicity in neuroblastoma cells and synergise strongly with buthionine sulfoximine

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ABSTRACT

In a wide range of neuroblastoma-derived lines oxovanadium compounds such as bis(maltolato)oxovanadium(IV) (BMOV) are cytotoxic. This is not explained by oxidative stress or inhibition of ion channels. Genotoxicity is unlikely given that a p53 response is absent and p53-mutant lines are also sensitive. Cytotoxicity is inhibited by N-acetyl cysteine and glutathione ester, indicating that BMOV action is sensitive to cytoplasmic redox and thiol status. Significantly, combining BMOV with glutathione synthesis inhibition greatly enhances BMOV-induced cell death. This combination treatment triggers high AKT pathway activation, highlighting the potential functional importance of PTP inhibition by BMOV. AKT activation itself, however, is not required for cytotoxicity. Oxovanadium compounds may thus represent novel leads as p53-independent therapeutics for neuroblastoma.

Introduction

Neuroblastoma is the most common and deadly extracranial solid tumour of infancy, accounting for approximately 10% of paediatric cancers [1,2]. Two potential therapeutic targets are *MYCN*, which is frequently amplified in high-grade tumours, and *ALK*, a receptor tyrosine kinase (RTK) oncogene identified in both familial and sporadic neuroblastoma [3,4]. Both ALK and MYCN act through AKT [5], a common consequence of activation of phosphotyrosine signalling. Phosphotyrosine signalling is jointly regulated by protein tyrosine kinases and protein tyrosine phosphatases (PTPs), with PTPs being key positive and negative modulators of this signalling [6–8]. With over 100 PTP family members, increasing numbers are known to play direct roles in tumour cell biology [7,9–11]. While historically regarded as tumour suppressors, a third of the tyrosine phosphatome may positively contribute to cancer cell survival and therapeutic resistance as shown in HeLa cells [12], and moreover there are recently-defined examples of specific tumour-supporting PTPs [13–15].

Although PTPs and their effectors are an emergent source of therapeutic targets in cancer, there is still relatively little documented research on their significance in neuroblastoma. Our own studies on neuroblastoma cells have demonstrated that oxovanadium compounds, which are broad inhibitors of PTP enzymes [16], induce differentiation and senescence in specific neuroblastoma cell lines [17]. Oxovanadium-based chemicals have long been of interest in cancer biology, with several studies demonstrating their anti-proliferative and anti-survival properties in tumour-derived cell lines [18]. They can also suppress tumour growth and have chemopreventive properties [14,19–22]. Inside cells, oxovanadium complexes exist largely in an equilibrium between vanadyl V(IV) and vanadate V(V) states, with V(V) being associated with PTP inhibition [23]. This V(IV)/V(V) equilibrium can also catalyse ROS generation through Fenton-like reactions and interactions with NADPH [14,19,24]. Many tumour cells are thought to exist in a state of sub-lethal oxidative stress and are sensitive to redox-based therapeutic approaches [14,25–29]. When oxovanadium (IV/V) is used at high levels, ROS generation and DNA damage may thus underlie some of its anti-tumour cell potential [18,21,30–32]. Interestingly, low concentrations of vanadate can be growth stimulatory under conditions of high cell density, but inhibitory at concentrations over 50 μM [33]. This may relate to the ROS levels generated. However, the importance of oxovanadium compounds as direct PTP inhibitors has also been demonstrated in several anti-cancer and anti-diabetic models [19,21,34]. The debate therefore continues as to whether PTP inhibition or oxidative stress or a combination underpin the anti-cancer effects of

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oxovanadium (IV/V). The ability of oxovanadium (IV/V) to inhibit some ion channels may also need to be factored in [23].

In this study we demonstrate that numerous neuroblastoma cell lines suffer a cytotoxic response to vanadate and the organometallic derivative bis(maltolato)oxovanadium(IV) (BMOV). Here we explore the specificity and potential cytotoxic mechanisms of these compounds in neuroblastoma cells. Our data indicate that neither oxidative stress nor ion channel blockade appears sufficient to explain the observed cytotoxicity, and the process is not dependent upon p53. The correlation of AKT activation and BMOV cytotoxicity indicates that PTP inhibition is occurring and may be necessary. Concurrent blockade of glutathione synthesis not only further enhances AKT activation, but also greatly increases BMOV cytotoxicity in neuroblastoma cells in culture. Oxovanadium chemistry may thus be exploitable for the development of novel neuroblastoma therapeutics and should also advance our understanding of survival-promoting PTP enzymes in paediatric tumours.

Materials and methods

Cell culture and treatments

Cells were maintained at 37 °C/5% CO_2. SKNSH were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich), 1% penicillin/streptomycin (P/S), 10% fetal bovine serum (FBS) and 2 mM L-glutamine. LAN-1, LAN-5, KCNR, IMR32, SKNAS, SKNDZ and N206 cells were cultured in RPMI 1640+GlutaMAX™ (Invitrogen), 10% FBS and 1% P/S, in some cases with added 25 mM HEPES pH 7. Mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified essential medium (DMEM), 1% P/S and 10% FBS. Sodium orthovanadate (VA), all-*trans* retinoic acid and Bis(maltolato)Oxovanadium IV (BMOV) were from Sigma-Aldrich. BMOV was also a gift from Prof John McNeill. Chemicals used: U0126 and LY294002 (Cell Signalling technologies); MK-2206 (Cambridge Bioscience); PI103 and rapamycin (Cayman biosciences); BSO, N-acetyl-L-cysteine, catalase and reduced glutathione ethyl ester (Sigma-Aldrich)); ouabain (Sigma-Aldrich); thapsigargin (Cambridge Bioscience).

Immunocytochemistry

Cells were plated onto 13 mm coverslips coated with poly-L-lysine and fibronectin. After fixing in 4% paraformaldehyde in PBS, cells were pre-blocked with PBS/1% BSA/0.05% triton. Cleaved caspase-3 antibody (Cell Signalling) in incubation buffer (PBS/3% BSA/0.05% triton) was added for 1 hour, followed by secondary antibody (Dako). Coverslips were mounted with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting solution (Dako).

Immunoblotting

Cells were lysed in ice-cold 1% Triton X-100; 50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM proteinase inhibitor cocktail [Roche], 1 mM sodium orthovanadate, 10 mM sodium fluoride, 25 mM sodium pyrophosphate. Lysates were transferred to polyvinylidene diflouride membranes after gel electrophoresis and blocked with 5% milk powder (Marvel) overnight. Primary and secondary antibodies were added for 1 hour each. Luminescent signal was developed using ECL plus (Amersham Biosciences and Thermo Scientific). Primary antibodies were from: Cell Signalling (phospho-Akt Ser473, ab4060; Akt, ab9272; S6 ribosomal protein, ab2217; phospho S6 ribosomal protein Ser 235/236, ab2211; p38 MAPK, ab9212; phospho p38 MAPK Thr 180/Tyr 182, ab9211; phospho-4E-BP1 Thr37/46, ab22855); Millipore (Phospho p44/42 MAPK (Erk1/2; Thr202/Tyr204) (9106 S), p44/42 MAPK (Erk1/2) (9102), and PTEN (07–1372)); Santa Cruz (Anti-p53 (SC6243)); Novus Biologicals (LC3 (nb100-2220)); Sigma-Aldrich (anti-βActin (AC-74)). Secondary antibodies were from DAKO.

Propidium iodide staining for sub-G1 DNA content

Trypsinised cells were fixed in ice-cold 70% ethanol for 30 minutes, rinsed twice in phosphate-citrate buffer (0.2M Na_2HPO_4/0.1M citric acid) by centrifugation at 2000 rpm, and resuspended in 200 µl of 50 µg/ml propidium iodide in PBS and 50 µl RNaseA solution (100 µg/ml in distilled water). Cells were analysed using a BD™ LSRII flow cytometer system (Beckman-Dickson Biosciences). A maximum of 10,000 events were collected per sample. Data were analysed using Flo-jo V8 software.

Detection of intracellular ROS and glutathione

Dihydrorhodamine (DHR) 123 (Cayman Biosciences) detected relative levels of intracellular ROS. Cells were seeded overnight in 96 well plates at high density ($5x10^4$) then treated with chemicals for 4 hours. Cells were washed with warmed Hanks balanced salt solution (HBSS), incubated with 10 µM DHR 123 in fresh HBSS for 30 minutes at 37 °C, then washed with HBSS and fluorescence was immediately read using a microplate reader at an excitation/emission ratio of 492/520. Monochlorobimane (MCB; Sigma-Aldrich) was used for the detection of total intracellular glutathione (GSH). Cells were treated with BSO and/or BMOV for 24 hours, then treated with 50 µM MCB. During this treatment period there are no morphological changes in the cells, or increases in cell death. Fluorescence was read immediately at an excitation/emission ratio of 426/490. Readings were taken every 10 minutes to confirm linear reactions. Final values at 60 minutes were then taken, background signals subtracted, then values normalised against the untreated cells at 100%.

Analysis of autophagy

Autophagy was detected by immunoblotting for LC3-II, where autophagy was measured by the relative change in band distribution from the upper (LC3-I) to lower (LC3-II) bands. LC3-II turnover was blocked using lysosomal peptide inhibitors pepstatin A (Sigma-Aldrich) and E64d (Enzo life sciences).

Results

BMOV induces cytotoxicity in neuroblastoma cells

Oxovanadium compounds, in combination with retinoic acid, induce differentiation and senescence in SKNSH, SH-SY5Y and LAN-5 [17]. We found that VA did not significantly increase apoptosis (as judged by sub-G1 content) in SKNSH, SH-SY5Y, or several unrelated cell lines (Fig. 1A). LAN-5, however, underwent increased cell death. Prompted by this, we tested further lines based on their varied status of MYCN amplification and p53 mutation (Table 1). We found that KCNR, LAN-5 and IMR32 underwent robust cell death with either VA (Fig. 1B) or the organometallic derivative bis(maltolato)oxovanadium(IV) (BMOV) (Fig. 1C). BMOV also killed N206 cells and subsequent studies showed that other lines were sensitive (Supplementary Fig. S1). SKNAS and SKNDZ are resistant to BMOV-induced differentiation [17], and also cytotoxicity with 10 µM BMOV (Fig. 1C).

We focused our subsequent studies on BMOV as it has higher bioavailability and is less toxic *in vivo* than VA [34]. BMOV increased sub-G1 content and caspase-3 activation in KCNR, N206, IMR32 and LAN-5 (Fig. 1C, Supplementary Fig. S3B). This was partially abrogated by the pan-caspase inhibitor zVad-FMK, as shown in KCNR cells (Fig. 1D), indicating that cytotoxicity is at least partly driven by apoptosis. Neither primary mouse embryonic fibroblasts (MEFs) nor SKNAS cells exhibited increased sub-G1 content after BMOV treatment (Supplementary Fig. S3A and B), even at 100 µM BMOV on MEFs (not shown). Thus compared to non-neuroblastoma cells, oxovanadium cytotoxicity shows some selectivity towards a subset of neuroblastoma cell lines.

From the pattern of BMOV sensitivity it appeared that high MYCN level might correlate with BMOV sensitivity. However subsequent analysis of MYCN-inducible lines showed that high MYCN expression was not sufficient to impart BMOV sensitivity (Supplementary Fig. S2).

Oxovanadium compounds can inhibit ion channel ATPases [23]. To assess if this underlies BMOV cytotoxicity, cells were treated with the Na^+/K^+ channel blocker ouabain and Ca^{++} channel blocker

Table 1
Neuroblastoma cell lines used.

Cell line	MYCN	p53
LAN-5	A	wt
KCNR	A	wt
IMR32	A	wt
N206	A	mut
SK-N-AS	NA	mut
SK-N-DZ	A	mut
SH-SY5Y	NA	wt
SK-N-SH	NA	wt

MYCN gene amplified (A) or non-amplified (NA). p53 protein is wild type (wt) or mutant (mut).