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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).

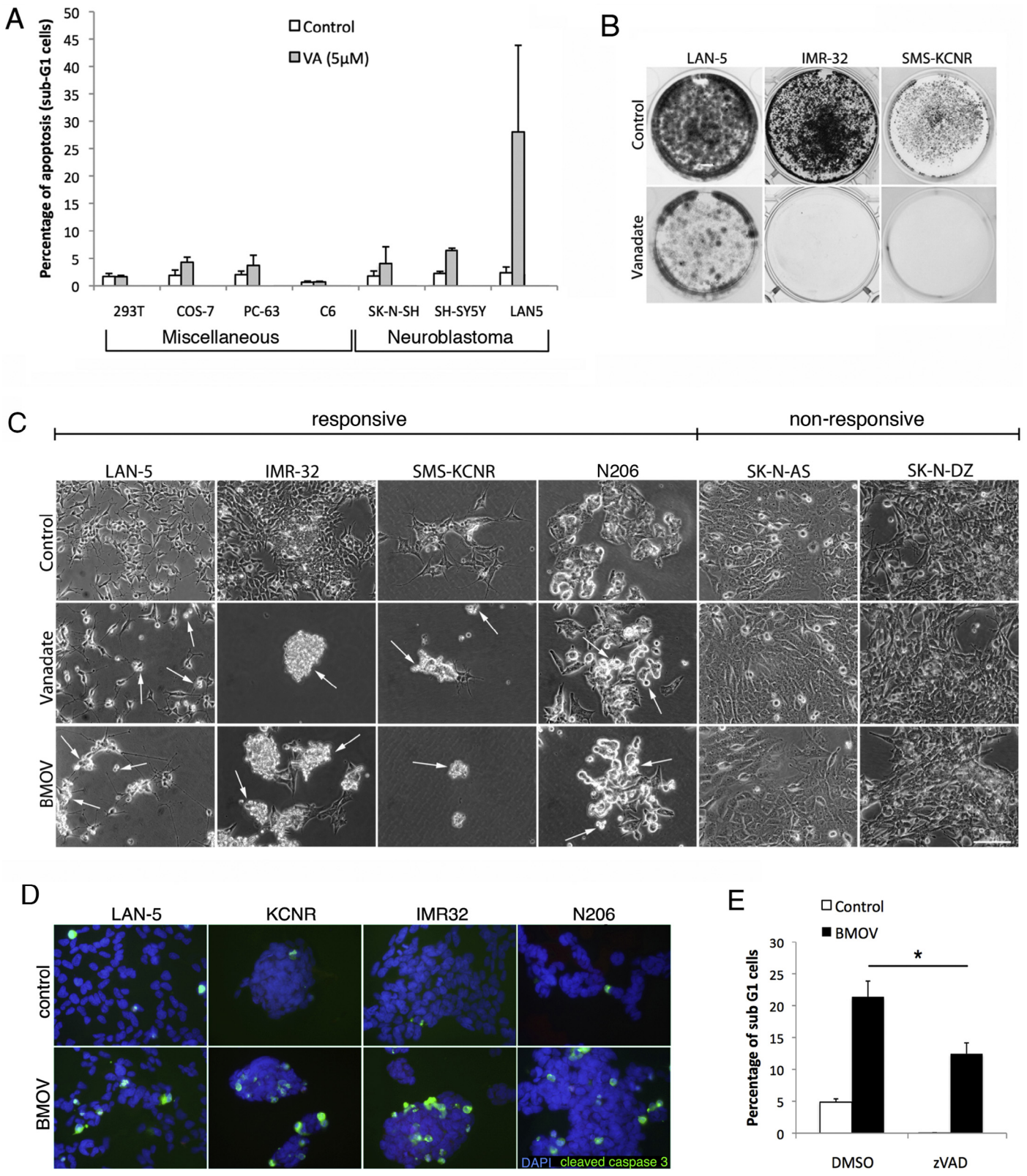

Fig. 1. Cytotoxicity of oxovanadium compounds in neuroblastoma cells. (A) Neuroblastoma cell lines and unrelated cell lines were treated with vanadate (VA) at 5 μM for 6 days and subjected to sub-G1 analysis. Only LAN-5 exhibited significant evidence of apoptosis. (B) LAN-5, IMR32 and SMS-KCNR were treated for 6 days with VA, then crystal violet stained. VA killed IMR32 and KCNR effectively, and partially killed LAN-5. (C) Six neuroblastoma lines treated with either VA (5 μM) or BMOV (10 μM) for 3–6 days. The cells are either oxovanadium-sensitive (LAN-5, IMR-32, KCNR, N206) or -resistant (SKNAS and SKNDZ). Arrows indicate dead and dying cells. Scale bar = 10 μm. (D) Immunofluorescence study of BMOV-treated cells (48 hours, 10 μM) using activated-caspase 3 antibody, highlighting apoptotic cells. (E) KCNR cells were treated with the pan-caspase inhibitor Z-vad-FMK (20 μM) during 48 hours of BMOV (10 μM) treatment. Z-vad-FMK reduces BMOV-induced cell death.

thapsigargin. Although these induced cell death, they did so in both BMOV-resistant (SKNAS, SKNDZ), and BMOV-sensitive cells (N206, KCNR, IMR32, LAN5, LAN-1, SKNBE(2)), between 50 nM and 250 nM (Fig. 2A). SKNAS and SKNDZ were more sensitive to ouabain than BMOV-sensitive N206. Moreover, SKNDZ and SKNSH underwent cell death with thapsigargin treatment just as readily as did BMOV-sensitive cell lines. The phenotypic responses to 10 μM BMOV therefore cannot readily be explained by inhibition of either Na^+/K^+ or Ca^{++} channel ATPases.

BMOV-induced cytotoxicity is dependent on an oxidised redox state

Many cytotoxic drugs increase ROS levels in tumour cells, or reduce the cell's capacity to withstand ROS damage [26,35]. An oxidised, cellular redox state can affect the oxidation state of vanadium, favouring V(V), the most inhibitory state for PTPs and ATPases. To test the potential requirement of an oxidising environment for BMOV-driven apoptosis, cells were treated with thiol-based ROS scavenger N-acetyl-L-cysteine (NAC). Low levels of NAC led to improved cell survival in BMOV-treated LAN-5 (Fig. 2B). NAC also reduced cell death in KCNR, albeit requiring higher levels of NAC (5 mM) (Fig. 2C). To assess the dependence of BMOV cytotoxicity on thiols such as GSH, we co-treated cells with GSH ethyl ester. This largely abolished BMOV-induced cytotoxicity in KCNR cells (Fig. 2D). BMOV-induced cytotoxicity thus requires a cytoplasm that encourages an oxidised thiol status.

BMOV cytotoxicity is enhanced by depletion of cellular glutathione

Two predictions arise from the above data. First, BMOV is likely to reduce cytoplasmic levels of major cellular antioxidants such as glutathione (GSH), by generating oxidised GSSG which is then exported and lost. Second, an experimental reduction in thiol-based, antioxidant defences should enhance BMOV's cytotoxicity. Using monochlorobimane (MCB) to detect total GSH, BMOV did indeed reduce GSH by 10–25% in LAN-5, N206, IMR32 and SKNAS over 24 hours (Fig. 2E). To test the second prediction, we co-treated cells with the GSH synthesis inhibitor BSO and BMOV. BSO has been used in clinical trials as an enhancer of anti-cancer drugs in various cancers including neuroblastoma [36–38]. BSO alone could deplete GSH by 10–40% in N206, IMR32, LAN-5 and SKNAS (Fig. 2E). Combination treatment of BSO+BMOV induced a further, significant reduction in GSH in LAN-5, N206 and IMR32 and a similar trend in SKNAS.

In our hands 10 μM BSO exerted little if any toxicity in most neuroblastoma cell lines. Exceptions included SKNDZ, killed by concentrations over 100 μM, and LAN-1, sensitive to 10 μM and above, in agreement with other studies [39]. Surprisingly, when 10 μM BSO was combined with 10 μM BMOV in BMOV-sensitive cells, this induced complete or nearly complete loss of cell survival over three days. This cytotoxicity was much greater than with either chemical alone, indicating a synergistic response (Fig. 3A and B**)**. This correlated with an enhanced sub-G1 response as shown in KCNR (Fig. 3C). A similar 100% cell death response was observed in the normally BMOV-resistant cell line SKNDZ (Supplementary Fig. S1), and a 50% response in SKNAS (Fig. 3A and B). Similar cytotoxicity could be generated at lower BMOV concentrations, by compensatory increases in BSO (Supplementary Fig. S6). Interestingly, SKNSH and SH-SY5Y, which differentiate in response to BMOV [17], are relatively resistant to this chemical combination (Supplementary Fig. S6). Similarly, monolayer cultures of the glioma line T98G, and MEFs, were resistant (Fig. 3A and B). Therefore BMOV cytotoxicity, when enhanced by BSO, exhibits promising efficacy and maintains some cell-type specificity in neuroblastoma-derived cell lines.

The ability of tumour cells to form 3D spheroids in semi-solid media reflects their transformed phenotype and resistance to anoikis. Neuroblastoma cells seeded in Methocel™ showed increased sensitivity to BMOV and BSO, and the combination treatment was highly effective at preventing colony formation in all cell lines tested (Supplementary Figs. S4 and S5).

BMOV/BSO stimulation of AKT is not necessary for cytotoxicity

It is possible that BSO enhances the cytotoxicity of BMOV by augmenting its chemical actions, for example by increasing V(V) in cells, leading to greater PTP inhibition. As an indication of this, we might expect to see elevated RTK signalling and increased phospho-AKT in combination-treated cells. Indeed this was observed, with much greater AKT activation in IMR32, LAN-5 and KELLY cells, and to a lesser degree with KCNR (Fig. 4A). We have seen related, strong AKT activation in our studies of combination BMOV/retinoic acid treatment on neuroblastoma cells [17]. In that study, hyperactivation of AKT was necessary in part for driving senescence in SKNSH and SK-SY5Y cells. Here we have similarly asked whether the high phospho-AKT arising from BMOV+BSO was necessary for the observed cytotoxic response. IMR32 cells were treated with BMOV+BSO along with the specific AKT inhibitor MK-2206. At 1 μM, MK-2206 efficiently blocked AKT phosphorylation (Fig. 4B), but it did not alter the ability of BMOV or the combined treatment to induce cell death (Fig. 4C). A similar result was seen with N206 cells (data not shown). Thus although BMOV and BSO can stimulate signalling downstream of RTKs in neuroblastoma cells, non-AKT-dependent pathways must be necessary to generate the cytotoxic synergy.

BMOV cytotoxicity and the AKT/mTOR axis

The stimulation of AKT by BMOV and BSO is not necessary for cytotoxicity. Instead, this AKT stimulation may be a pro-survival bystander effect of BMOV treatment, countering qualitatively distinct anti-survival effects of BMOV. We therefore investigated the PI3K-mTOR axis further, asking whether the inhibition of PI3K enzymes could enhance the BMOV cytotoxicity and whether BMOV has other downstream influences over mTOR signalling.

BMOV stimulates AKT and should therefore activate mTOR. BMOV does stimulate S6K1, as evidenced by increased phosphorylation of substrate S6 ribosomal protein (Fig. 5A). The class 1 PI3K inhibitor LY294002 (LY29) suppressed this S6 phosphorylation as expected. LY29 also stimulated an increase in BMOV-driven apoptosis in LAN5 and KCNR cells (Fig. 5B). LY29 was not, however, an ideal inhibitor in these cells, since it led paradoxically to an increase in AKT phosphorylation, exaggerated by BMOV (Fig. 5A). This is most likely due to suppression of mTORC1 and the well known negative feedback loop from S6 Kinase 1 [40–42]. To bypass this problem, we used the narrower spectrum PI3K/mTOR inhibitor PI103 [43] and the mTOR inhibitor rapamycin (at a level that should inhibit both mTORC1 and mTORC2). Both inhibitors suppressed BMOV-induced phospho-AKT (Fig. 5C and G). PI103 also caused an increase in BMOV-driven apoptosis (Fig. 5D). Neither of the combined treatments of BMOV/LY29 and BMOV/PI103 induced toxicity on MEFs (Supplementary Fig. S1C).

Concurrent inhibition of PI3K alongside BMOV treatment thus enhances cytotoxicity. We examined whether BMOV has any further influence over the mTOR signalling pathway in these cells. AKT activates mTOR, inhibiting autophagy and stimulating protein translation [44,45]. After PI103 or rapamycin treatment, autophagy activation occurred as judged by increased LC3II (Fig. 5E). 4E-BP1 phosphorylation was also suppressed (Fig. 5F). Although BMOV did not affect basal LC3II levels, it blocked induction of LC3II by PI103 and Rapamycin (Fig. 5G**)**. This was not the result of mTOR reactivation, since PI103-induced dephosphorylation of 4E-BP1 still occurred with BMOV (Fig. 5F). Even though BMOV induces pS6 phosphorylation, it does not induce phosphorylation of p4E-BP1 (Fig. 5F).

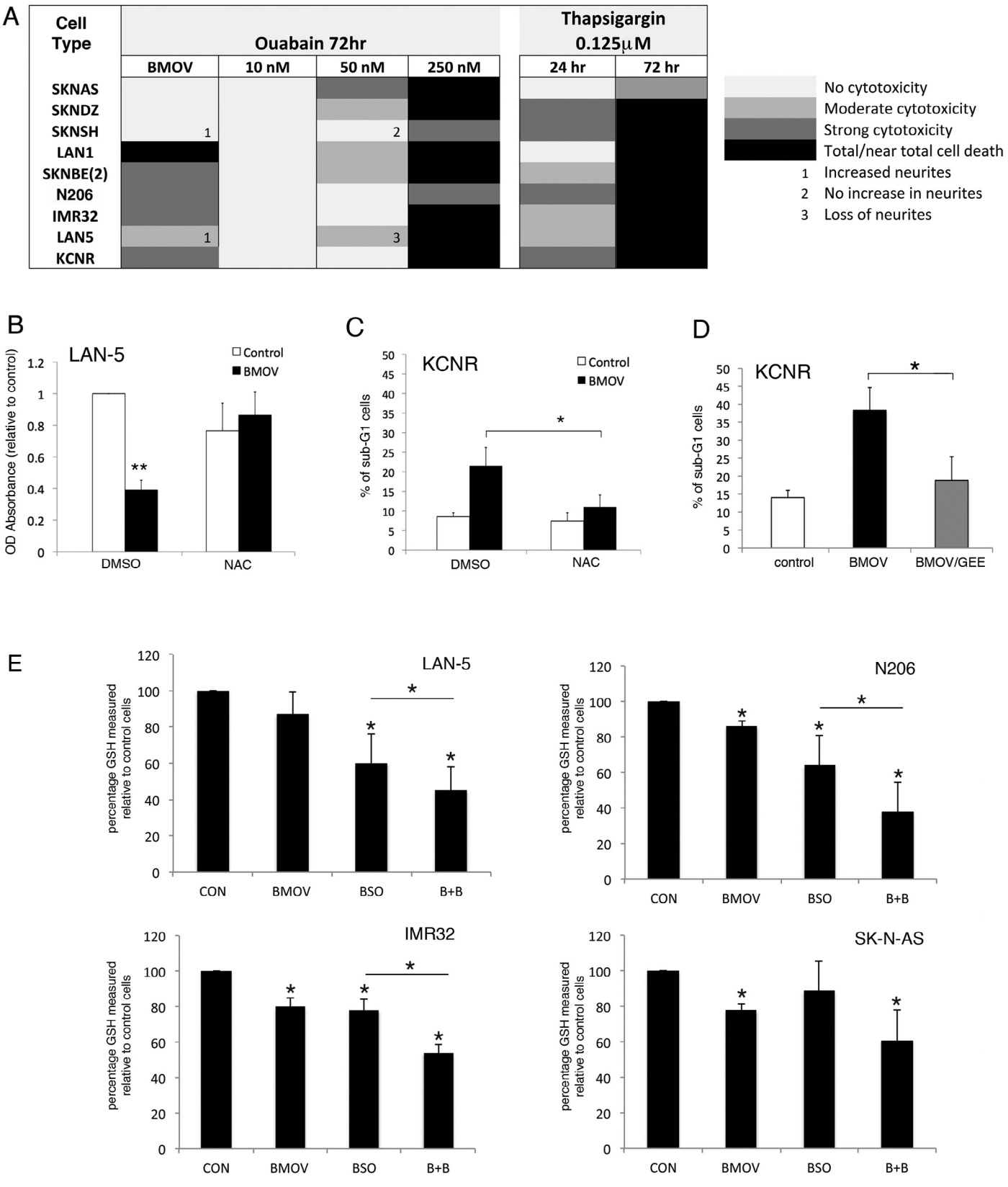

Fig. 2. BMOV toxicity does not correlate with ion channel blockade, but is thiol dependent. (A) Comparison of cytotoxicity pattern induced by ion channel blockade and BMOV. The Na^+/K^+ channel inhibitor ouabain and Ca^{++} channel blocker thapsigargin were used for up to 72 hours, then cells were categorised qualitatively for neurite formation and relative level of cell death. These were compared to the same cell lines treated with 10 μM BMOV for 72 hours. (B) LAN-5 were grown for 3 days with or without BMOV (10 μM), and with or without N-acetyl-L-cysteine (NAC) (100 μM). Cells were crystal violet-stained and quantified. (C) Treatment of KCNR cells with 5 mM NAC for 24 hours; sub-G1 analysis. (D) Sub-G1 analysis of KCNR cells treated overnight with BMOV with or without GSH ethyl ester (GEE, 2 mM; *$P < 0.05$, SD are shown). (E) Glutathione levels are suppressed by BMOV and BSO. IMR32 and LAN-5 were treated with BMOV (10 μM), BSO (10 μM) and the combination for 18 hours (n = 3). N206 and SKNAS were treated with BMOV for 14 hours following 12 hour pre-treatment with BSO (n = 5). GSH was measured using monochlorobimane and values were normalised against untreated cells. *$P < 0.05$ (SD shown).

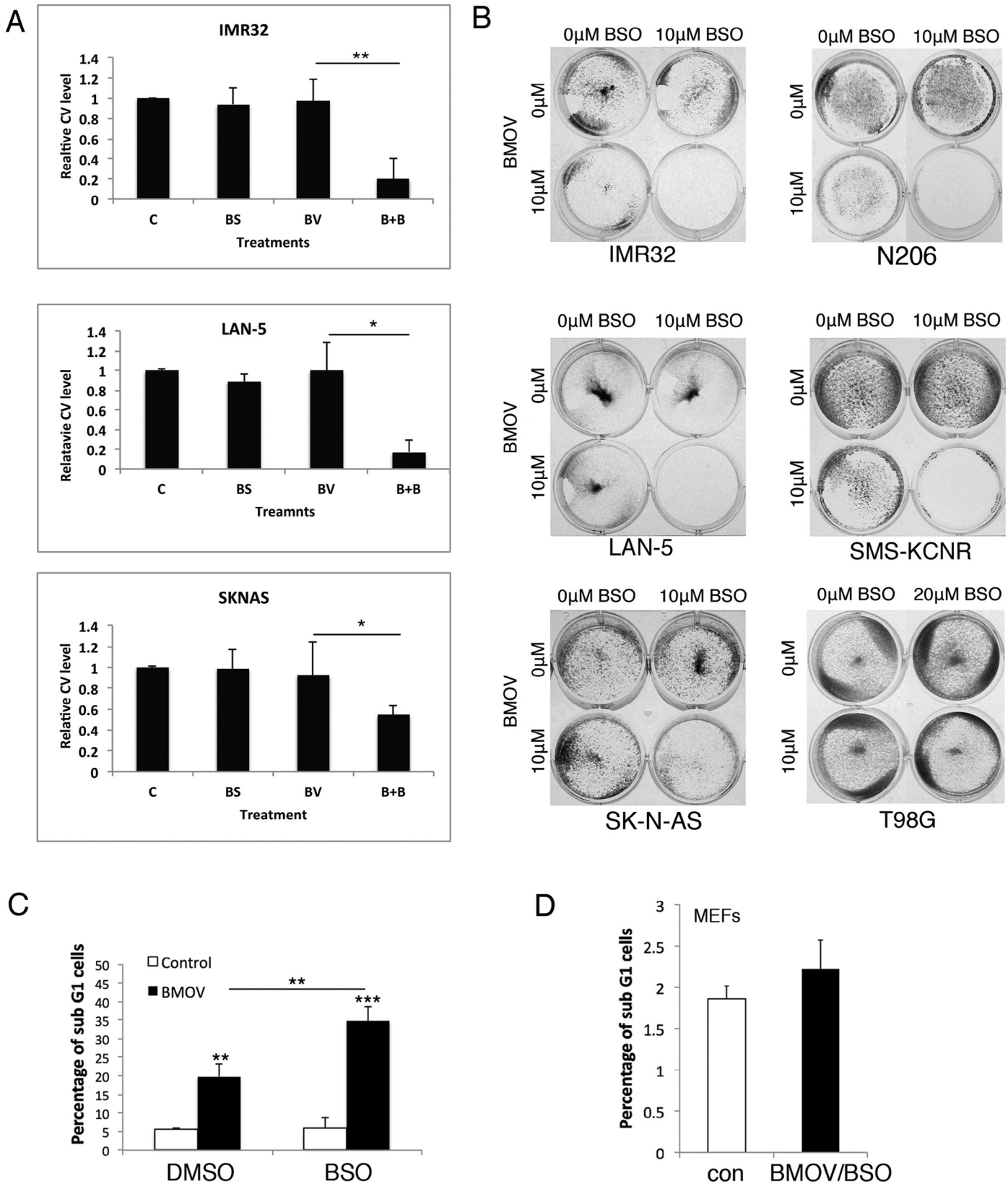

Fig. 3. Enhancement of BMOV cytotoxocity by glutathione blockade. (A) and (B) Cells were treated with BMOV (10 μM), BSO (10 μM) or a combination, for three days, then fixed and crystal violet (CV) stained. CV quantitation is shown in A (n = 3). BSO has no significant effect on cell viability; BMOV had mild effects in these experiments; in combination most cells are killed. SKNAS cells showed a 50% loss of viability after combination treatment, while T98G glioma cells were resistant. (C) Sub-G1 analysis on KNCR cells after 3 days BMOV+BSO. (D) Primary mouse fibroblasts are not affected by BMOV+BSO. SD are shown; $^{*}P < 0.05$, $^{**}P < 0.005$.

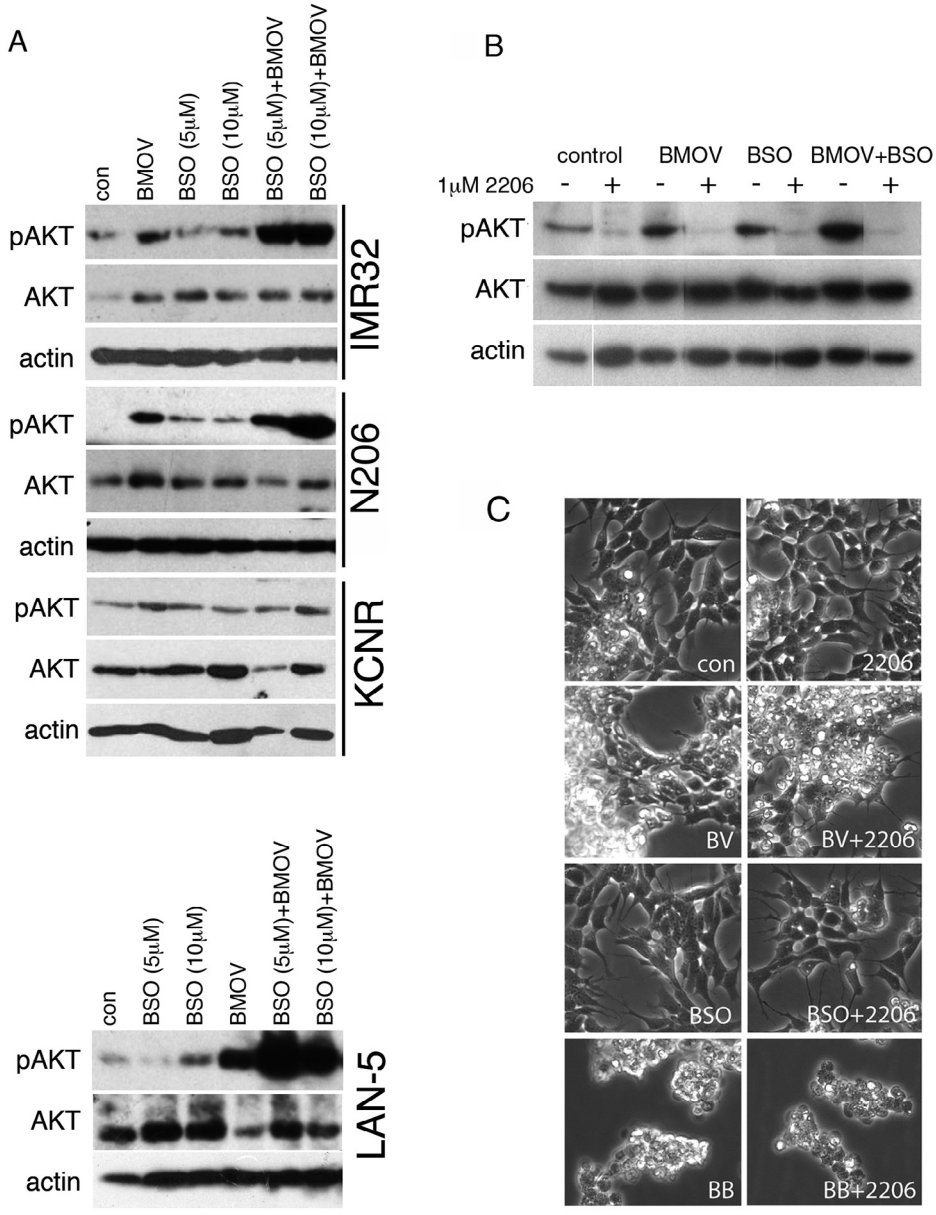

Fig. 4. AKT activation by BMOV+BSO. (A) In IMR32, N206, LAN-5 and KCNR, 10 μM BMOV stimulates AKT after 24 hour treatment, whereas BSO has little or no effect. Combination treatment stimulates much greater AKT activation in IMR32, N206, LAN-5, with a lesser response in KCNR. Note the different loading order with LAN-5. (B) IMR32 cells were chemically treated for 24 hours, with and without 1 μM MK-2206 (untreated, con; BMOV+BSO, BB). MK-2206 inhibits AKT. (C) Similar cells to (B) were treated for 3 days. MK-2206 does not block the cytotoxicity of BMOV or BMOV+BSO.

In fact in most KCNR experiments BMOV suppressed 4E-BP1 phosphorylation, while concurrently activating AKT (Fig. 5G). BMOV may therefore interfere with translational control even in the presence of elevated phospho-AKT and pS6.

BMOV and oxidative stress

We have described how BMOV and BSO can strongly stimulate pathways through AKT, most likely reflecting their PTP-inhibitory role. However, the concomitant cytotoxicity is not dependent on AKT activation and so non-AKT pathways must be involved. Another chemical action of oxovanadium inside cells is the generation of reactive oxygen species (ROS) [14,19,24]. High ROS levels can lead to non-specific oxidative stress and cellular damage, leading to cell death. We have therefore assessed the potential role of ROS and oxidative stress in BMOV-induced neuroblastoma cell death.

ROS were examined directly with DHR-123 in live cells. Using N206 and the more BMOV-resistant SKNAS, the DHR-123 dye indicated similar, modest increases in ROS with single BMOV or BSO treatments. In combination treatments there was no significant, further increase in ROS (Fig. 6A and B). Moreover, the similar ROS patterns in both N206 and SKNAS showed that ROS levels were not predictive of BMOV sensitivity. ROS production in cells can also involve increased hydrogen peroxide levels. To test whether hydrogen peroxide production might underlie BMOV-induced signalling and apoptosis, we added catalase to the media of treated cultures. Catalase can for example block vanadate-induced apoptosis in JB6 mouse fibroblasts [46]. In neuroblastoma cells, however, catalase

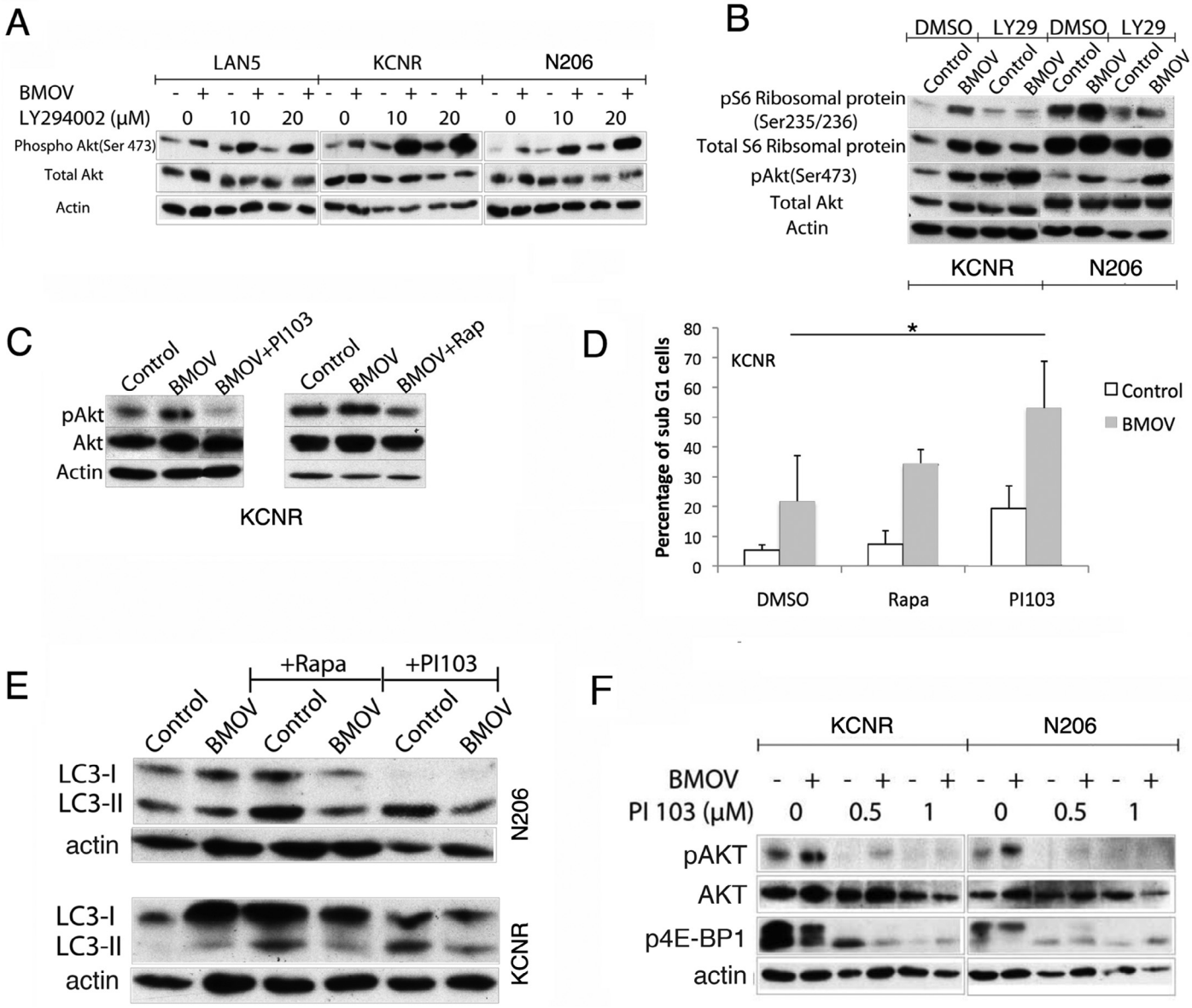

Fig. 5. BMOV, AKT activation and mTOR signalling. (A) 10 μM BMOV treatment over 24 hours increases S6 ribosomal protein phosphorylation and AKT phosphorylation. LY294002 (5 μM) inhibits S6 phosphorylation, but concurrently activates AKT. (B) Sub-G1 analysis of LAN-5 and KCNR cells after 48 hr treatment with BMOV (10 μM), and LY294002 (5 μM), separately or combined. Combined treatment significantly increases apoptosis. (C), treatment of KCNR with either PI103 (500 nM) or rapamycin (100 nM) for 24 hours suppresses BMOV-induced AKT activation. (D) Sub-G1 analysis of KCNR cells treated for 24 hours with BMOV +/– 100 nM rapamycin or 500 nM PI103. (E) Autophagy-related LC3-II band (lower) is induced by rapamycin (200 nM) and PI103 (1 μM) over 15 hours in both N206 and KCNR. BMOV inhibits this stimulation of LC3-II. (F) Analysis of reduced phosphorylation of 4E-BP1 and AKT over 24 hours by PI103, in the presence or absence of BMOV.

did not improve cell survival in N206 cells (Fig. 6C) or in LAN-5 (not shown). In addition, catalase did not alter phospho-AKT stimulation by BMOV and BSO, indicating that this aspect of BMOV's chemical action is unaffected (Fig. 6D). This also holds true for phospho-ERK activation by BMOV (data not shown). These data therefore do not readily support a role for high ROS production, or peroxide specifically, in BMOV-induced cytotoxicity.

We next examined p38MAPK status and PTEN oxidation levels, to assess oxidative stress. BMOV did not reproducibly increase the expression or phosphorylation of p38 stress-sensitive kinase (Fig. 6E). The lipid-specific PTP enzyme PTEN is particularly sensitive to oxidation in neuroblastoma cells following growth factor activation [14,47]. PTEN is not however oxidised after BMOV or BMOV+BSO treatment in either KCNR or SKNSH cells (Fig. 6F). Finally, although it is known that high oxovanadium concentrations can trigger a genotoxic response in some cells [46], 10 μM BMOV did not trigger p53 signalling in LAN-5, IMR32 or KCNR (all express wild type p53) (Fig. 6G). Moreover, BMOV killed p53-mutant N206 cells (Figs. 1 and 3B), LAN-1 cells (Supplementary Fig. S1) and SKNBE(2) (not shown). Therefore neither the activation of p53, nor non-specific oxidative damage apparently underlies BMOV cytotoxicity. Thus although the key mechanism(s) remains to be determined, we have ruled out several of the more expected mechanisms for the highly cytotoxic action of BMOV+BSO in neuroblastoma cells.

Discussion

This study provides the first demonstration that oxovanadium-based chemicals can effectively induce cell death in a broad range of neuroblastoma tumour-derived cell lines, in part through

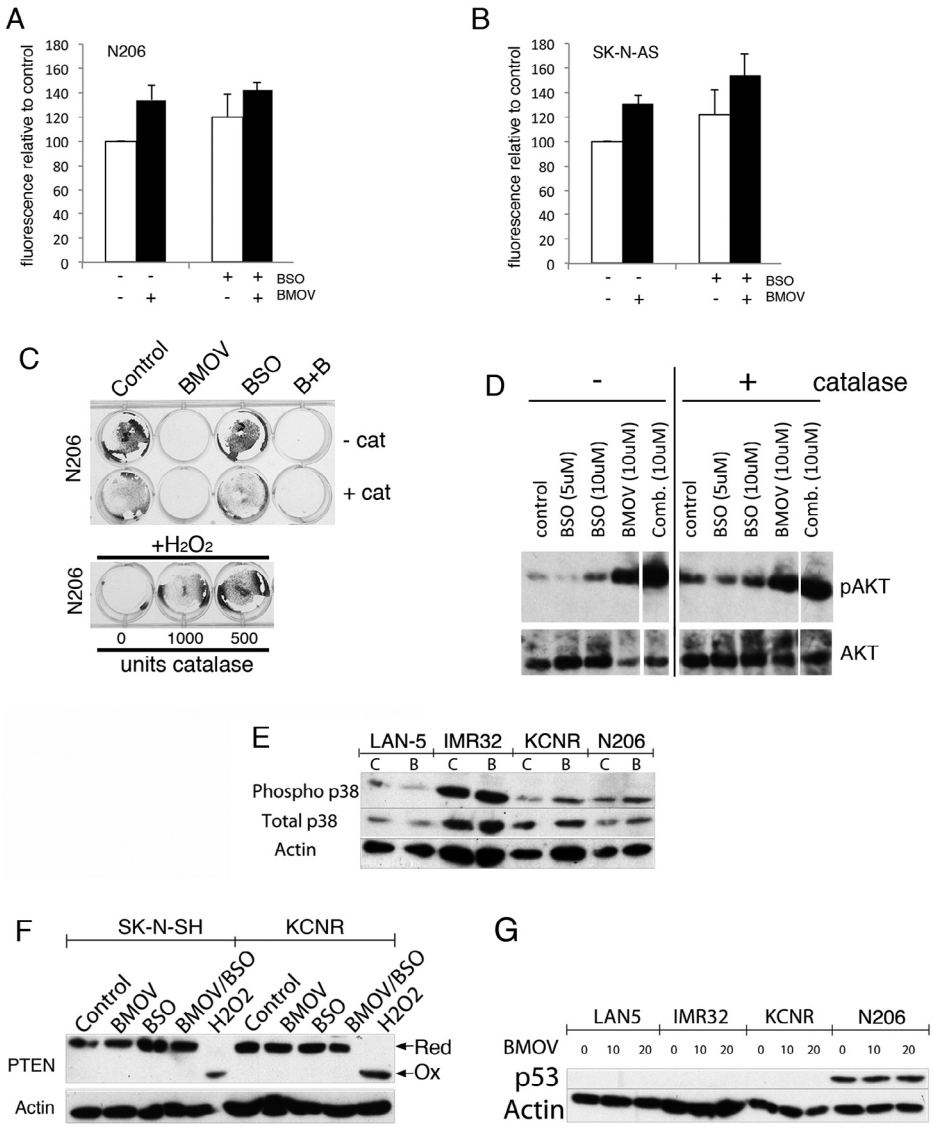

Fig. 6. Oxidative and genotoxic stress do not explain BMOV and BSO cytotoxicity. N206 (A) and SKNAS (B) were treated with or without BSO (10 μM) for 24 hours, then for 4 hours with BMOV (10 μM). ROS levels were measured in live cells using DHR, normalised to control (n = 3). (C) Test for catalase suppression of BMOV cytotoxicity. N206 cells were treated with 1000 units per ml of catalase (+cat) for three days, with and without BMOV and BSO. Separate wells were treated with a cytotoxic level of 100 μM H_2O_2 +/– catalase (500 units or 1000 units per ml) to confirm catalase protection of cells. (D) LAN5 cells were treated for 24 hours as in (C) and lysates immunobloted for phospho-AKT, AKT and actin. (E) LAN-5, IMR32, KCNR and N206 cells were treated with BMOV for 24 hours and p38 phosphorylation assayed. (F) Cell lysates were electrophoresed under non-reducing conditions to reveal the faster-migrating, oxidised PTEN protein in peroxide-treated cell samples (5 minutes of 2 mM H_2O_2). Oxidised PTEN is not observed after 24 hours with BMOV (10 μM), BSO (10 μM) or the combination in KCNR or SKNSH. (G) Cells treated with 10 μM or 20 μM BMOV for 24 hours do not induce p53 protein.

apoptosis (summarised in Supplementary Table S1). In the vanadate state, oxovanadium compounds are potent, broad inhibitors of PTPs in their V(V) oxidised state. The V(IV):V(V) redox equilibrium also catalyses ROS generation, but our data do not support oxidative stress nor ROS production as directly underlying BMOV cytotoxicity at the concentration we are using it at. Nevertheless, cytotoxicity can still be blocked by reducing thiols, which may discourage PTP-inhibitory V(V) or reactivate oxidised PTPs. In contrast, BMOV-induced cell death is greatly enhanced by its combination with glutathione synthesis inhibitor BSO. Significantly, the cytotoxic mechanism of BMOV does not rely on p53 activation, making this potentially relevant for p53-mutated neuroblastomas found commonly at relapse. The novel chemical combination of oxovanadium compounds with BSO could thus generate interesting therapeutic possibilities for neuroblastoma.

Conventional chemotherapy often triggers oxidative damage in tumour cells, but the side effects can be severe. To circumvent this and minimise therapeutic resistance, one might target a non-oncogene-dependent 'Achilles' heal' of cancer cells. Sub-lethal oxidative stress is one of these [48], with cells relying on heightened antioxidant defences to defend themselves against endogenous ROS [25,26]. This "primed" state of stress can potentially be harnessed in redox-related therapeutic approaches [35]. PTPs are particularly redox-sensitive enzymes, having ROS-sensitive active site cysteines [41,47,49–51]. Some PTP members may also act non-oncogenically to promote tumour cell survival and therapeutic resistance [12], and some may also act oncogenically in neuroblastoma [41,52]. If tumour cells are oxidatively primed with partial PTP inhibition already, they may be susceptible to more direct PTP inhibition treatments such as with BMOV.

Oxovanadium compounds are broad specificity PTP inhibitors, reversibly inhibiting tyrosine-specific and dual-specificity enzymes [24,53,54]. An oxidising tumour cell environment should encourage the V(V) PTP inhibitory state of vanadium [55]. We have recently found that many of our untreated cell lines are in fact in a relatively oxidised state as assessed with RedoxSensor dye (Stoker, unpublished work). Our data indicate that by imposing a reducing state on cells, BMOV-induced cytotoxicity is less effective. This supports the need for an oxidising redox state in promoting oxovanadium action. Such an oxidising environment does not however go as far as to induce clear oxidative stress. How does BSO therefore enhance BMOV cytotoxicity? This could be either direct or indirect. Oxidised, active site thiols of PTPs are directly targeted by GSH, leading to enzyme reactivation. Together with the propensity of GSH to directly discourage the formation of PTP-inhibitory V(V) [56,57], this may explain why BSO can generate a potent mixture with BMOV to potentially block critical members of this enzyme family.

Although vanadate can inhibit some other classes of enzymes, these are not directly thiol dependent [58]. For example, vanadate can inhibit some ion channel ATPases, but this does not appear to underlie BMOV cytotoxicity here in neuroblastoma cells. This may be further supported by research showing that, in contrast to BMOV ([17] and the present study), ouabain induces ERK in SKNAS [59] and p53 in SHSY5Y (but not phospho-AKT) [60], as well as cell cycle arrest in SHSY5Y, KELLY/N206 and SKNAS [61]. Moreover, unlike BMOV, AKT phosphorylation is suppressed by thapsigargin in SHSY5Y cells [62]. This further supports the parsimonious explanation for BMOV action here in neuroblastoma cells as being in part through critical PTP inhibition. Inhibition of cellular PTPs by BMOV can readily explain the elevation in phospho-AKT found in neuroblastoma cells, most likely through negative regulators of RTK signalling such as PTPN2, PTPRJ or PTEN [63]. The very high phospho-AKT levels seen with BMOV+BSO treatment are not, however, required for a cytotoxic response. This contrasts with the requirement for high phospho-AKT for senescence of SKNSH cells during BMOV+retinoic acid treatment [17] and apoptosis in other systems [64]. Although AKT activation by BMOV (+BSO) is a likely result of PTP inhibition, it therefore appears to be a "bystander" cell survival response, since blocking PI3K signals alongside BMOV treatment increases the cytotoxic response. This may even be exploitable therapeutically, given that PI3K and AKT are currently high profile targets for neuroblastoma treatment.

There are some further, interesting aspects to BMOV's effects on the PI3K/mTOR axis. In KCNR cells BMOV surprisingly causes dephosphorylation and probable activation of 4E-BP1 downstream of mTOR, even though AKT is activated. Moreover, BMOV can conditionally block autophagic induction during PI103 or Rapamycin treatment. Autophagy is a potentially protective mechanism following oxidative stress in SH-SY5Y neuroblastoma cells [65–67] and is a target in cancer trials [68,69]. How BMOV prevents LC3II activation remains to be understood, but a number of its potential PTP targets can influence autophagy both positively and negatively [70]. Translational regulation and blockade of autophagy could therefore be further facets of BMOV's cytotoxic arsenal.

Our finding that oxovanadium compounds can kill neuroblastoma cells without activating p53 was unexpected. Vanadate's documented influence over p53 is rather complex, however, since 100 μM vanadate suppresses radiation-induced p53 activation, but not p53-independent apoptosis in MOLT-4 leukaemia cells [71]. In JB6 mouse fibroblasts, 100 μM vanadate induces p53-dependent apoptosis, suppressible by culturing in catalase [46]. In the neuroblastoma cells in this study, 10 μM BMOV does not trigger these p53 events. BMOV must therefore require a non-p53-dependent pathway(s) to kill neuroblastoma cells. This could be advantageous given that relapse of neuroblastoma often occurs alongside disruption of the p53 pathway [72,73].

Why are neuroblastoma cell lines so susceptible to oxovanadium? This may reflect either their particular pattern of PTP dependence, or sensitised metabolic or oxidised state. Is the cytotoxicity specific for neuroblastoma? Although we have found resistance in fibroblasts, some non-tumour cells and T98G glioma cells, a preliminary screen of paediatric glioma lines shows that a minority can also be killed by BMOV and this is increased by BSO (Stoker, unpublished work). Oxovanadium may therefore be of interest in brain cancers, as has recently also been reported [74].

In conclusion, we have demonstrated that oxovanadium compounds are effective cytotoxic agents in neuroblastoma tumour-derived cell lines, irrespective of p53 status. This complements our previous work showing oxovanadium-induced differentiation and senescence in SK-N-SH, SH-SY5Y and LAN-5 [17]. Moreover, low levels of the redox modulator BSO can synergise with BMOV to become highly cytotoxic in 80% of tested cell lines. Although we do not know the key cytotoxic mechanism(s) yet, we have already ruled out a number of the potentially non-specific mechanisms of BMOV action. Our data remain most consistent with BMOV being able to trigger cell death through a non-AKT-dependent pathway downstream of PTP inhibition. If correct, this would indicate the existence of critical, survival-promoting PTPs in neuroblastoma cells and we are currently searching for such enzymes. This would be concordant with growing evidence of PTPs as pro-oncogenic effectors and novel therapeutic targets [12,41,52]. The finding that a novel combination of BMOV plus BSO can amplify cytotoxicity in neuroblastoma and some other cancer cell lines may encourage a reassessment of oxovanadium compounds as anti-tumour agents.

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Conflict of interest

None.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.11.039.

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