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Sodium tungstate modulates ATM function upon DNA damage

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ABSTRACT

Both radiotherapy and most effective chemotherapeutic agents induce different types of DNA damage. Here we show that tungstate modulates cell response to DNA damaging agents. Cells treated with tungstate were more sensitive to etoposide, phleomycin and ionizing radiation (IR), all of which induce DNA double-strand breaks (DSBs). Tungstate also modulated the activation of the central DSB signalling kinase, ATM, in response to these agents. These effects required the functionality of the Mre11–Nbs1–Rad50 (MRN) complex and were mimicked by the inhibition of PP2A phosphatase. Therefore, tungstate may have adjuvant activity when combined with DNA-damaging agents in the treatment of several malignancies.
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1. Introduction

Despite great advances in our understanding of the biology of cancer and new approaches using gene or targeted therapy, cancer treatment still primarily consists of surgery when possible, followed by chemo- and radio-therapy. The development of new anticancer drugs is an inefficient and costly process that takes around 10–15 years and an investment of over $1 billion USD for an average new molecule to enter the market. In addition, data suggests that the overall success rate for oncology products in clinical development is ~10% [1,2].

The ultimate goal of chemo- and radio-therapy is to selectively kill cancer cells while sparing healthy ones and minimizing side effects. The most effective chemotherapeutic agents and radiotherapy strategies induce DNA damage. DNA double-strand breaks (DSBs), one of the most cytotoxic types of DNA damage, trigger signalling pathways that lead to cell death or senescence when repair fails. The cellular response to DSBs is governed by the Mre11–Rad50–Nbs1 (MRN) complex (Mre11–Rad50–Xrs2, MRX, in budding yeast) which is a sensor of DSBs, and the Ataxia-telangiectasia mutated (ATM) kinase, a key transducer of the signalling response [3]. While DSB-based therapies effectively kill many cancer cells, they are also toxic to healthy dividing cell populations like those in the bone marrow and gastrointestinal tract. Advances in chemotherapy will depend on finding the key differences between tumoral and normal cells and taking advantage of them to selectively kill certain cells.

One approach to this end is the identification of adjuvants that can increase the effectiveness of current therapies [4–7]. Ideally, an adjuvant should be non-toxic, easy to administer and have known pharmacological properties.

Tungstate can mimic, with a small toxicity profile, most of the effects of insulin, both in long- and short-term treatments [8,9]. Sodium tungstate administered orally, normalizes glycemia, without causing hypoglycemia, in many animal models of type 1 and 2 diabetes [9–12]. It can also significantly reduce weight gain and adiposity by increasing energy dissipation and fatty acid oxidation rates in obese rats [13]. The molecular mechanisms of tungstate action remain poorly understood, but available data suggests that they involve the inhibition of phosphatases [14–17]. This notion has been linked to the capacity of this molecule to regulate translational control pathways [18]. While recent data in human patients showed no significant anti-obesity activity of tungstate in phase II clinical trials in the conditions and doses assayed [19] its low toxicity profile and potential to act on diverse physiological processes make it a potential adjuvant in therapeutic protocols.

Here we report that micromolar concentrations of tungstate can increase the toxicity of DSB inducing agents in both yeast and

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human cells, by potentiating the natural response to the damage that these agents elicit. The molecular mechanism underlying this effect involves the MRN complex and ATM pathway and is modulated by the PP2A phosphatase. These results indicate that tungstate should be considered a potential adjuvant for radio- and chemo-therapy.

2. Materials and methods

2.1. Yeast growth assays

Yeast strains are described in Table 1. Standard methods for yeast culture and manipulations were used [20].

For proliferation assays, exponentially growing cultures in liquid YPD were 20-fold diluted in the specified conditions. Growth at 28.5 °C was monitored in a Bioscreen C analyzer (Thermo Labsystems) as $Abs_{420–580}$ measured every 30 min for 48 h. Data represented are the means of triplicates of each mutant and condition versus untreated control.

2.2. Cell lines culture

Human HeLa cell line and wild type and $Mre11^{ATLD1/ATLD1}$ mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) with 4.5 g/L glucose and supplemented with 10% Fetal Bovine Serum (FBS) and 1 mM glutamine. Wild type and $Mre11^{ATLD1/ATLD1}$ MEFs have been previously described [21].

2.3. MTT viability assays

We followed the procedure published in [22]. Briefly, the day before treatment, the medium from cells in the logarithmic phase was removed and replaced by 0.5% FBS DMEM. In the case of etoposide and phleomycin (Sigma) cells were treated for 3 h, after which the medium was removed and substituted by fresh medium without drug. For ionizing radiation (IR), the treatment lasted the time required to reach the indicated doses. Tungstate at the indicated concentrations was always present in the medium from 1 h prior to challenge with DNA-damaging agents. The day after treatment, the medium was removed and cells were incubated with 1 mg/ml MTT (Sigma) in culture medium for 2–3 h. Dye was then extracted from the intact, viable cells with a solution of 0.1 N HCl and 10% Triton (v/v) in isopropanol. The absorbance of solubilized dye was then determined using a spectrophotometer equipped with a 570 nm filter. Quantification of the extracted MTT by spectrophotometry was normalized to mock-treated cultures. Statistical analysis was performed using a two-tailed *t*-test.

2.4. Immunoblotting

Cells seeded on p60 plates were scraped with 100 μl/plate of 25 mM HEPES, pH 7.3, 300 mM NaCl, 1.5 mM $MgCl_2$, 0,1% (v/v) Triton X-100, 10 mM NaF, 1 mM sodium ortovanadate, 17.5 mM β-glycerophosphate, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20 nM okadaic acid, 50 nM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinine, 10 μg/ml leupeptine and 10 μg/ml pepstatine. 20 μg of soluble protein extract was subjected to SDS–polyacrylamide gel electrophoresis and transferred to PVDF (Immobilon-P; Millipore) filters. Uniform gel loading was confirmed by Ponceau S staining of membranes after transfer. Phosphorylated ATM (S1981) and Smc1 (S957) were detected with antiphosphoprotein antibodies from Rockland. Antibodies against total ATM (Rockland) and Mre11 (Abcam) proteins were used to ensure the even loading of gels. Immunocomplexes were visualized by enhanced chemiluminescence detection (GE Life Sciences) using a HRP-conjugated secondary antibody. The figures show an experiment representative of at least two independent ones with essentially identical results.

Table 1 Yeast strains.

Name	Genotype	Reference
W303.1a	MATa leu2–3,112 his3–11,15 ura3–1 ade2–1 trp1–1 can1–100	[48]
Δ*mre11*	W303.1a mre11::HIS3	[49]
Δ*rad50*	W303.1a rad50::TRP1	[49]
Δ*xrs2*	W303.1a xrs2::LEU2	[49]
Δ*mrx*	W303.1a mre11::HIS3 rad50::TRP1 xrs2::LEU2	[49]

2.5. Lentivirus production and shRNA gene silencing

Human *MRE11A*, *NBN*/*NBS1* and *RAD50* genes were silenced in HeLa cells using the shRNA-based MISSION technology (Sigma) with lentiviral particles. TRCN0000039872 (*MRE11A*), TRCN0000040135 (*NBN*) or TRCN0000040104 (*RAD50*) shRNAs were selected. Cells were incubated at 33 °C and medium was collected for 2 days. HeLa cells were infected with the produced lentiviruses and selected with 5 μg/mlpuromycin. As a control, an shRNA designed to silence mouse Glycogen Synthase (TRCN0000075796, noted as A8) with no effect on human cells, was used.

2.6. Colony formation assay

The sensitivity of cells to IR was determined by colony formation assay as described [23]. Briefly, logarithmic phase HeLa and MEF cultures were irradiated at the indicated gray (Gy) doses and reseeded for colony formation in triplicate. At 7–10 days after plating, colonies were visualized by crystal violet staining and counted. Colony numbers on plates receiving treatment were normalized to mock-treated cultures. Statistical analysis was performed using a two-tailed *t*-test.

2.7. Expression of recombinant proteins in HEK293T

HEK293T cells were seeded in p150 plates. When confluent, cells were transfected with 50 μg/plate of two pCDNA3-flag constructs: PP2A and PP5. The first day post-transfection the medium was replaced by fresh DMEM (Lonza) cell culture medium supplemented with 10% FBS. On the second day post-transfection, cell culture medium was removed, and 500 μl of cold lysis buffer (30 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 nM okadaic acid, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin) was added per dish. Cells were collected using cell scrapers and incubated in lysis buffer for 20 min in an orbital shaker at 4 °C and low speed. The extract was then centrifuged for 10 min at 5000×*g*. The supernatant was then collected. Next, 200 μl of anti-Flag agarose resin (Sigma) was added to the extract, and it was incubated overnight at 4 °C using an overhead tumbler. The following day cell extract with resin was centrifuged at 1000×*g* and 4 °C for 5 min. The resin was transferred to an Eppendorf tube, where it was washed once using 500 μl of cold wash buffer (30 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Nonidet P-40) and five times more with TBS. The resin was then incubated for 10 min with elution buffer (30 mM Tris–HCl, pH 7.4, 150 mM NaCl and 500 μg/ml flag peptide), and protein was eluted.

2.8. Protein phosphatase assays

Protein phosphatase activity using *p*-nitrophenylphosphate (pNPP) as substrate was determined essentially as described [24].

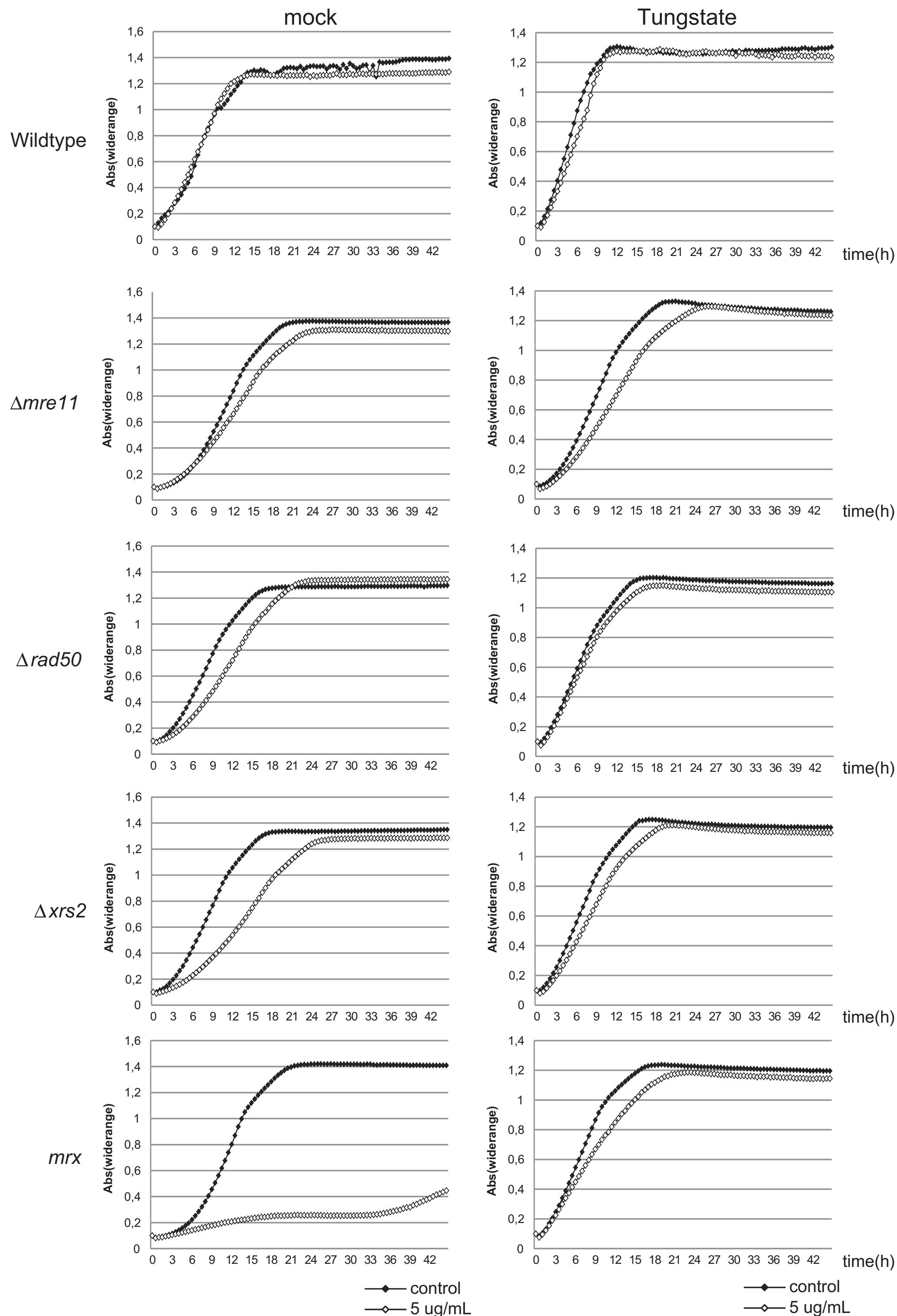

Fig. 1. Tungstate rescues proliferation of yeast MRX complex mutants upon DNA damage. Growth of W303.1a wild-type strain, Δ*mre11*, Δ*rad50* and Δ*xrs2* single mutants, and a MRX complex triple mutant with (open symbols) or without (black symbols) 5 μg/mL phleomycin in the presence or absence of 1 mM tungstate. Raw $Abs_{420-580}$ as means of the triplicates are represented.

The reaction buffer was 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 2 mM $MnCl_2$, and 0.2% (v/v) β-mercaptoethanol. Samples were incubated for 10 min at 37 °C, and then the reaction was stopped by adding 1% Tris (final concentration). For phosphatase inhibition assays, a range of concentrations of tungstate were incubated with the purified phosphatases for 10 min at 37 °C, prior to the addition of pNPP.

3. Results

3.1. Tungstate rescues proliferation of yeast MRX complex mutants upon DNA damage

Phleomycin is a glycopeptide antibiotic that belongs to the bleomycin family and causes DSBs in DNA. A *Saccharomyces cerevisiae*

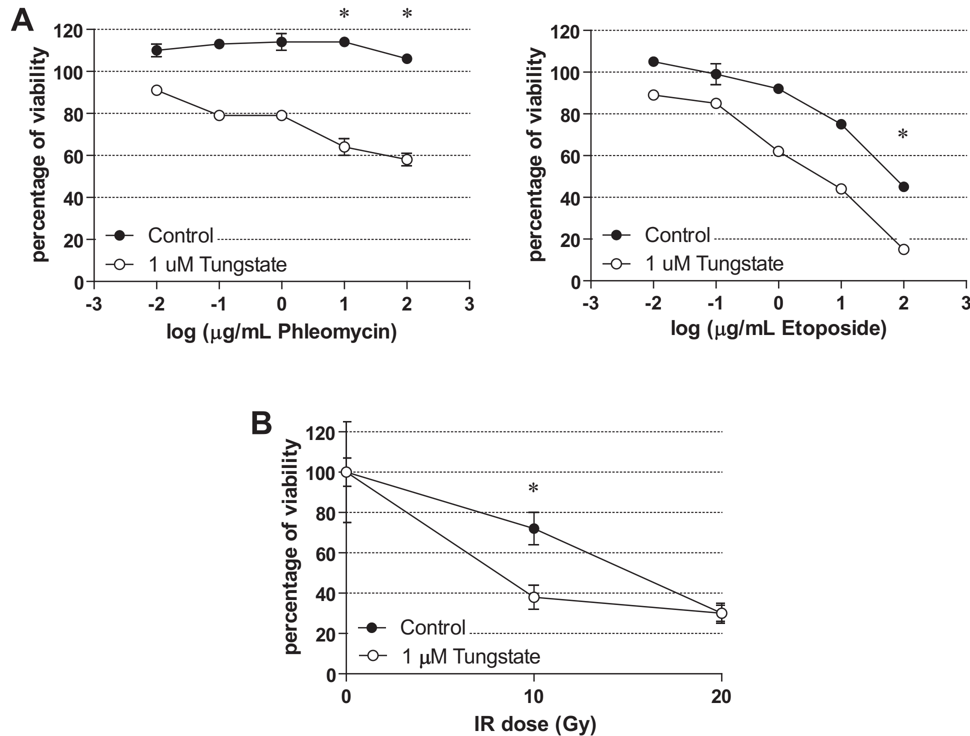

Fig. 2. Tungstate enhances the toxicity of DNA damaging agents in HeLa cells. MTT viability assays of HeLa cells: (A) incubated with increasing concentrations of phleomycin or etoposide ±1 μM tungstate. (B) Irradiated with 10 or 20 Gy in the presence or absence of μM concentrations of tungstate. $^{*}P < 0.05$.

W303.1a wild-type (*WT*) strain, Δ*mre11*, Δ*rad50* and Δ*xrs2* single mutants, along with the MRX complex triple mutant were subjected to treatment with 5 μg/ml phleomycin. We tested the effect of 1 mM tungstate on cell growth after phleomycin-induced DNA damage. As expected, all MRX mutants showed a higher sensitivity to phleomycin than the *WT*, specially the *mrx* triple mutant. Surprisingly, tungstate alleviated sensitivity to phleomycin in all mutant strains and had a dramatic effect on the triple *mrx* mutant (Fig. 1). However, despite the strong effect on cell growth, viability was not rescued (Suppl. Fig. 1). This result indicates that signalling downstream of the MRX complex is impaired by tungstate after a DNA damage challenge.

3.2. Tungstate enhances toxicity of DSB-inducing agents

Etoposide, phleomycin and IR all induce DNA damage, including DSBs [25–27]. The MTT assay was used to assess the decrease in HeLa cells viability caused by DSB-inducing agents in the presence of several concentrations of tungstate. Tungstate was added 1 h prior to the induction of DNA damage and maintained during and following treatment. For each drug, tungstate treatment augmented the toxicity of the DNA damaging agent in a dose dependent manner (Fig. 2).

3.3. Tungstate modulates the kinetics of ATM pathway activation

ATM phosphorylation and subsequent activation of its kinase activity is a hallmark of the DSB response [28] and Smc1 is a specific target of ATM phosphorylation [29]. HeLa cells were preincubated or not with 1 μM tungstate for 1 h and then challenged with 1 or 10 μg/ml etoposide. Samples were collected at several time points and immunodetection of active ATM was carried out using an antibody for the S1981 autophosphorylation site. Tungstate treatment enhanced the activation kinetics of ATM (Fig. 3A). A similar effect was obtained when HeLa cells (Fig. 3B) and MEFs (Fig. 3C) were pre-incubated or not with 1 or 10 μM tungstate for 1 h and then irradiated with 5 or 10 Gy. Samples were collected before and 3 and 6 h after irradiation. Again, more robust phosphorylation of ATM was observed in the presence of tungstate.

3.4. Depletion of the MRN complex blocks tungstate-induced enhancement of DNA damage

The Mre11–Nbs1–Rad50 (MRN) complex is a major component of the ATM pathway. MRN is a sensor of DSBs and is required to activate ATM and mediate its activation of targets including all the members of the MRN complex and SMC1 [30]. MRN complex constituents were individually depleted in HeLa cells by using shRNA lentiviral expression. We achieved around a 75% reduction in mRNA levels in every case, as measured by RT-qPCR (Suppl. Fig. 2). When each of the MRN components was silenced, thus compromising the function of the MRN complex, tungstate was unable to enhance etoposide-induced cell death (Fig. 4A). To confirm this observation, a colony formation assay was performed in HeLa cells and MEFs irradiated in the presence or absence of tungstate. Neither Mre11 shRNA-expressing HeLa cells (Fig. 4B), nor defective mutant *Mre11*$^{ATLD1/ATLD1}$ MEFs (Fig. 4C) were sensitive to the DNA-damage-enhancing activity of tungstate. Of note, Mre11 shRNA-expressing HeLa cells without tungstate treatment and WT cells with it showed similar sensitivity to etoposide. This effect is consistent with an impairment of Mre11 function.

3.5. Tungstate inhibits the ATM phosphatase PP2A in vitro

Tungstate inhibits several types of phosphatases [14–17], therefore it is reasonable to hypothesize that the inhibition of a protein phosphatase explains the effect of this compound on the modulation of ATM signalling. Protein phosphatase 2A (PP2A) and protein phosphatase 5 (PP5) are two of the main phosphatases involved in ATM dephosphorylation [31,32]. We used human PP2A and rat PP5

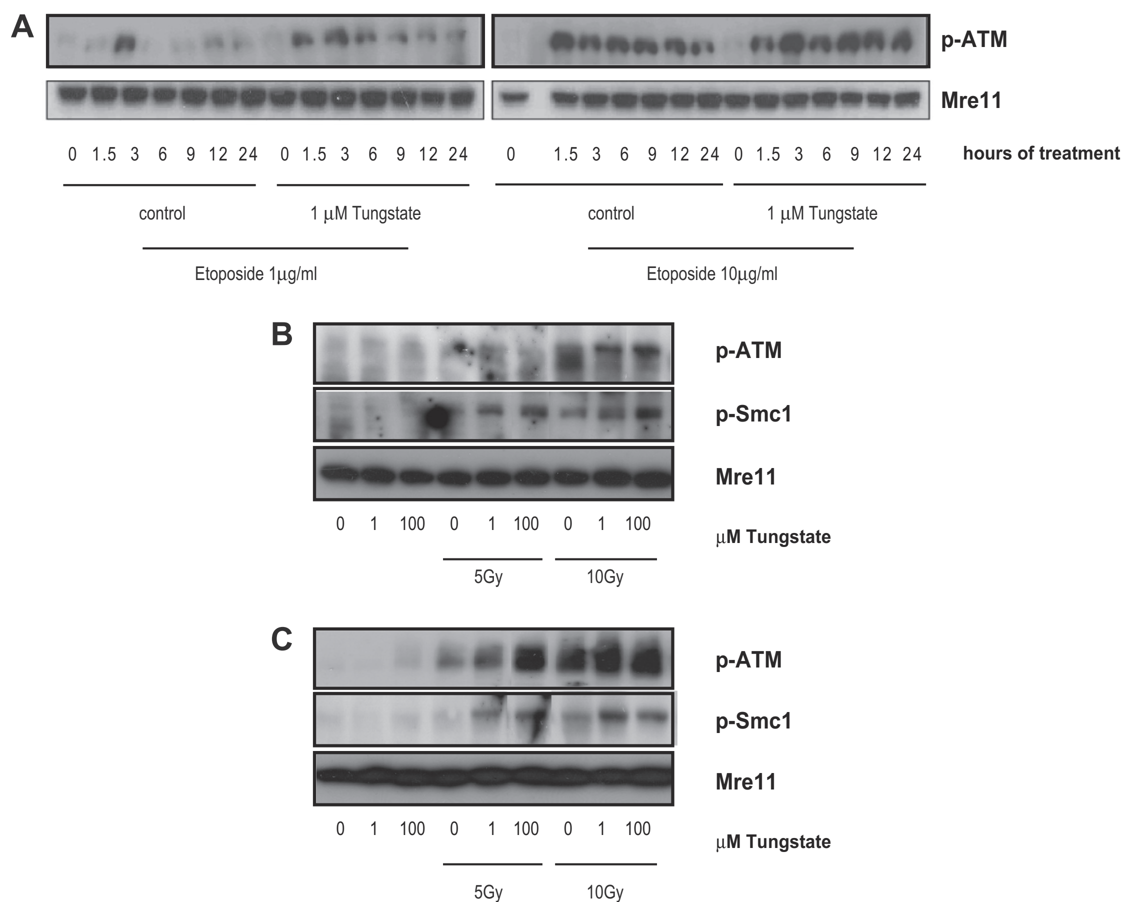

Fig. 3. Tungstate modulates ATM and Smc1 phosporylation kinetics upon DNA damage. (A) Immunodetection of phospho-ATM (p-ATM) in HeLa cells treated with 1 or 10 μg/mL etoposide for the hours indicated ±1 μM tungstate. (B) Immunodetection of p-ATM and phospho-Smc1 (p-Smc1) in HeLa cells irradiated with 5 or 10 Gy and with indicated concentrations of tungstate for 3 h. (C) Immunodetection of p-ATM and phospho-Smc1 (p-Smc1) in wild-type MEFs irradiated with 5 or 10 Gy and with indicated concentrations of tungstate for 3 h. Even loading of the gels was confirmed by immunodetection of Mre11 protein.

flag-tagged fusion proteins to evaluate in vitro the sensitivity of these phosphatases to tungstate. Tungstate inhibited PP2A (Fig. 5A) with an IC_{50} nearly 10-fold lower than that described for PP1 [18] and similar to the dose used on HeLa and MEFs. In contrast, tungstate was a poor inhibitor of PP5 (Fig. 5B).

3.6. In vivo inhibition of PP2A mimics the effects of tungstate on DNA damage signalling

We addressed whether the inhibition of PP2A phosphatase is involved in the role of tungstate on the modulation of the DNA-damage response (DDR) modulation. For this purpose, we treated HeLa cells with etoposide in the presence or absence of okadaic acid (OA), a potent inhibitor of PP2A, and to a lesser extent PP1 [33]. OA mimicked the cell responses to etoposide-induced DNA damage caused by tungstate (Fig. 6) as it increased cell death and modulated ATM phosphorylation.

4. Discussion

Our study shows that tungstate interferes with DDR signalling triggered by the MRN complex in yeast and mammals. Consistent with this, tungstate enhanced the toxicity of DSB-inducing agents in HeLa cells and MEFs. Tungstate modulated the kinetics of ATM activation. This effect appears to require a functional MRN complex, as depletion of any of its components blocked the effect. To our knowledge this is the first study to report that tungstate participates in the MRN-ATM response to DNA damage. Our results suggest the involvement of a negative regulator of the ATM pathway, which would be blocked by tungstate. Indeed, tungstate inhibited the ATM phosphatase PP2A in vitro. Accordingly, the PP2A inhibitor OA mimicked the tungstate-induced phenotype.

In budding yeast, tungstate alleviated the growth defect in MRX complex single mutants and, more dramatically, in the MRX triple mutant upon phleomycin-induced DNA damage. As these mutants are fully defective in DSB repair machinery but only partially defective in checkpoint signalling, this effect indicates that tungstate affects signalling downstream of MRX.

Accordingly, tungstate treatment enhanced the toxicity of a variety of agents that generate DSBs, such as phleomycin, etoposide and IR in a human cell line. Analysis of ATM activation and substrates such as SMC1 showed that the ATM signalling cascade was activated in these treatments and was affected by tungstate [3]. The MRN complex has diverse functions in DNA damage recognition [34], cell cycle checkpoint activation [35], non-homologous end joining [36] and telomere maintenance [37]. The MRN complex binds DSB soon after they are formed implicating it in DNA damage detection [38], thus acting upstream ATM and allowing its activation. The complex can tether linear duplex molecules [39], and it is able to bridge broken DNA ends or sister chromatids [40]. MRN function is complex as it also acts downstream of ATM to facilitate the phosphorylation of numerous substrates including Smc1 and Chk2 [3]. On the basis of our results, we hypothesize that action of tungstate on the ATM kinase is exerted upstream, at the

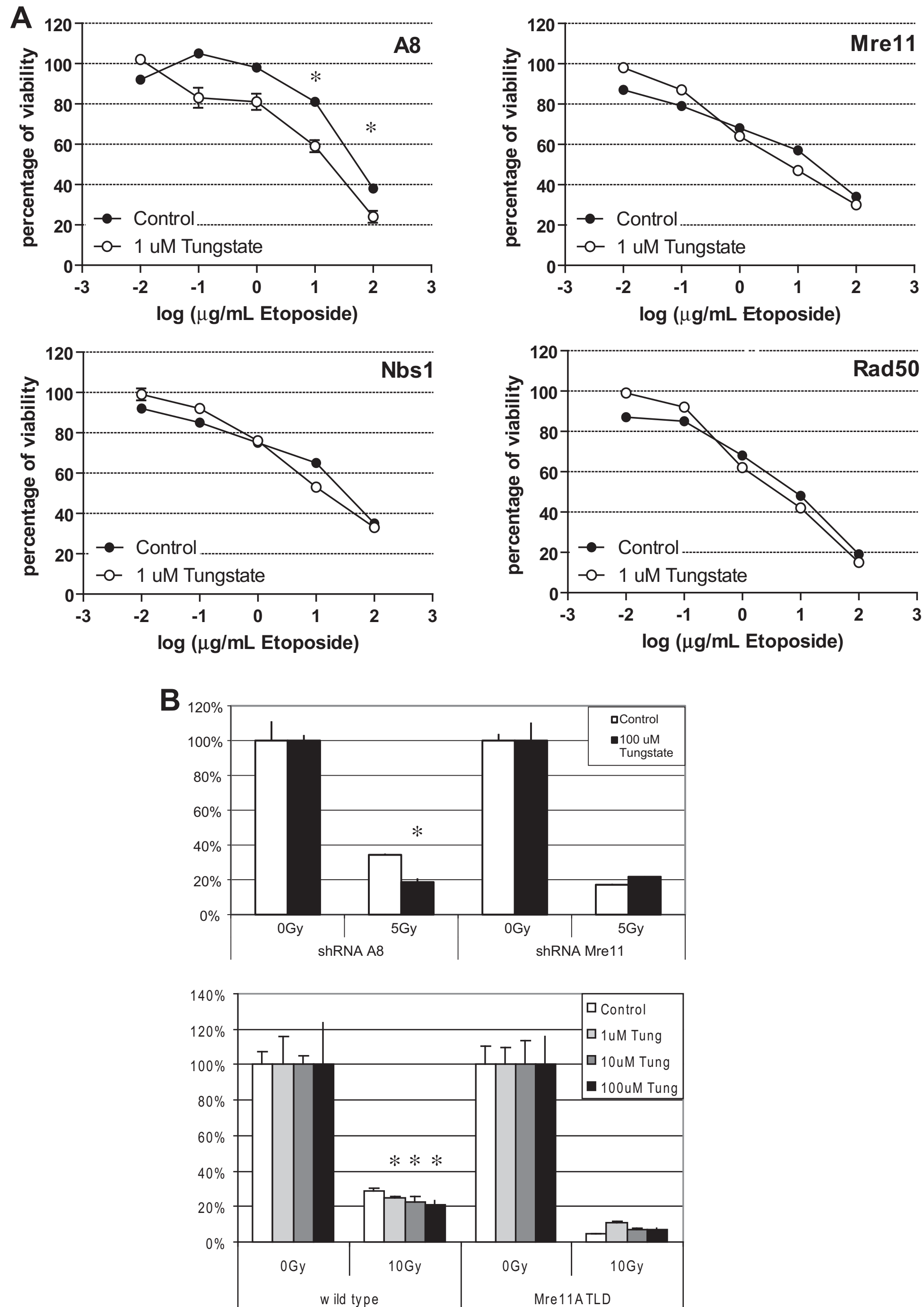

Fig. 4. Downregulation of the MRN complex abolishes the effect of tungstate on DNA damage response. (A) MTT viability assay of HeLa cells stably expressing shRNA for MRN complex constituents, treated with increasing concentrations of etoposide ±1 μM tungstate. (B) Colony formation assay of HeLa cells stably expressing control and Mre11 shRNA and irradiated with 5 or 10 Gy in the presence of increasing concentrations of tungstate. (C) Colony formation assay of wild type and Mre11$^{ATLD1/ATLD1}$ cells irradiated with 5 or 10 Gy in the presence of increasing concentrations of tungstate. *$P < 0.05$.

DNA damage site, or downstream, thus affecting the negative regulation of the kinase.

The intensification of the DDR by tungstate requires the MRN complex. Although inherited mutations in *MRE11A* and *NBN*/*NBS1* genes are responsible for the cancer-prone syndromes ataxia telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS), these essential genes are neither extensively mutated nor is their expression in tumors greatly reduced, as breast cancer data show [41,42]. Moreover, an intact MRN complex has been correlated with effective eradication of tumor cells by radio-therapy [43]. Tungstate action could take advantage of the frequent conservation of MRN function in tumors in order to improve the DDR and increase the cytotoxicity elicited by DNA damaging agents.

The inhibition of the PP2A phosphatase by tungstate seems to mediate ATM overactivation, as this effect was mimicked by OA treatment. Apart from ATM dephosphorylation [32], PP2A is implicated in the regulation of numerous signalling pathways and it may function as a tumor suppressor. Pharmacological modulation of PP2A activity has recently shown promising therapeutic activity for the treatment of cancer [44].

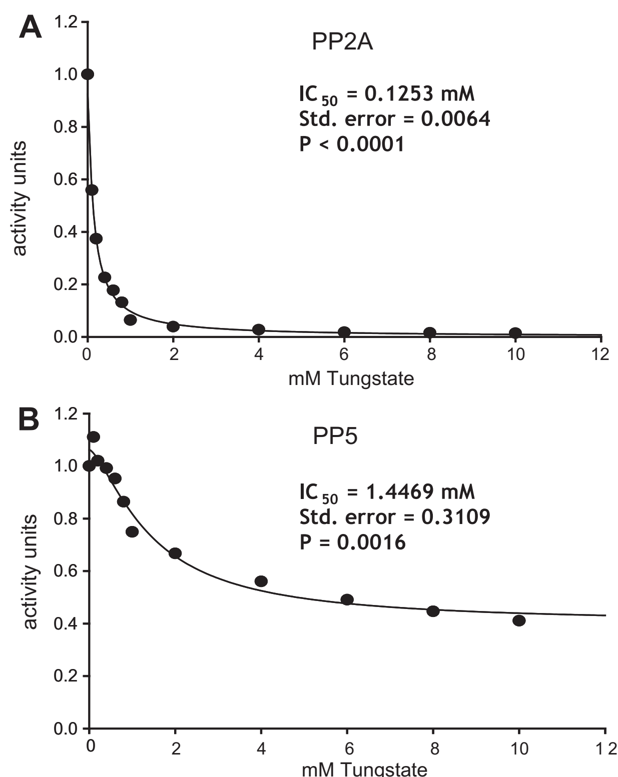

Fig. 5. In vitro inhibition by tungstate of phosphatases involved in ATM signalling. Protein phosphatase activity using *p*-nitrophenylphosphate as substrate in the presence of increasing concentrations of tungstate of: (A) human protein-phosphatase 2A; and (B) rat protein-phosphatase 5, expressed in and purified from HEK293T cells as explained in Section 2. IC_{50} data were calculated with Sigma-Plot software (Systat Software, Inc).

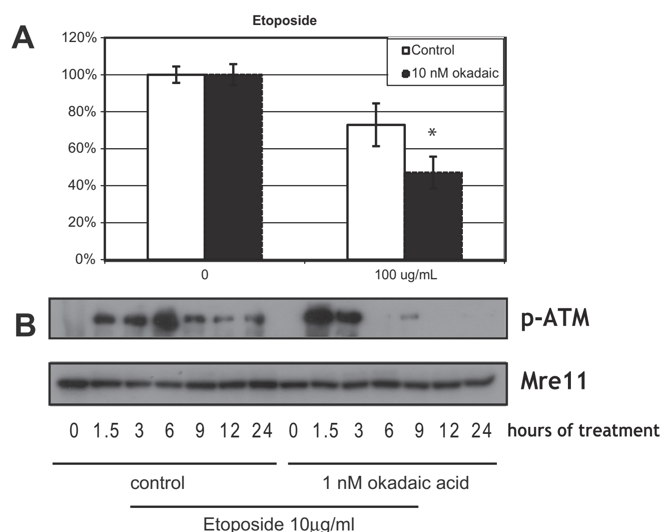

Fig. 6. Okadaic acid mimics the effects of tungstate on etoposide toxicity and ATM phosphorylation. (A) MTT viability assay of HeLa cells incubated with increasing concentrations of etoposide ±10 nM okadaic acid. $^{*}P < 0.05$. (B) Immunodetection of p-ATM in HeLa cells treated with 10 µg/mL etoposide for the hours indicated ±1 nM okadaic acid. Even loading of the gels was confirmed by immunodetection of Mre11 protein.

However, we cannot rule out the possibility of direct inhibition of Mre11 nuclease activity by tungstate. Mre11 contains sequence motifs equivalent to those of the PP1 catalytic site, recently described to be inhibited by tungstate [18], and they are important for the nuclease activity of the protein [45]. Although the contribution of this activity to DSB processing is controversial, the phosphatase inhibitory activity of tungstate could impair Mre11 nuclease activity during DDR and thus enhance cytotoxicity.

Apart from their pivotal role in responding and repairing extrinsically induced DNA damage, ATM and the MRN complex also participate in DNA-replication stress [46]. This event guarantees the fidelity of DNA replication as it can stop when DNA damage is detected. It is estimated that DNA damage occurs at a rate of 1000 to 1 000 000 molecular lesions per cell per day [47]. Whether tungstate affects this everyday process in a relevant manner deserves further investigation.

Tungstate shows anti-diabetic and anti-obesity effects in many experimental models. Unfortunately data obtained from phase II clinical trials do not support tungstate as a pharmacological agent for the treatment of obesity [19]. Nevertheless it has passed phase I trials, so data are available regarding its safety, tolerability and pharmacokinetics. Furthermore, the pathways potentially regulated by this compound, according to the literature, are multiple. For all these reasons, it is extremely attractive to explore new therapeutic potentials of tungstate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.04.003.

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