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Tyrosine phosphatase inhibitors combined with retinoic acid can enhance differentiation of neuroblastoma cells and trigger ERK- and AKT-dependent, p53-independent senescence

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Retinoic acid (RA)-induced differentiation therapy is partially successful in neuroblastoma treatment. We found that a novel combination of vanadium-based PTP inhibitors with RA induced extensive differentiation in neuroblastoma cells. In contrast to RA alone, this led to either permanent differentiation or senescence after 14 days of combined treatment followed by chemical removal. Senescence was dependent in part on synergistic AKT and ERK activation. p21 was also strongly induced, but in contrast to oncogene-induced senescence, p53 was not activated. Vanadium-based inhibitors thus serve strongly to enhance RA's ability to drive differentiation and a novel form of senescence in neuroblastoma cells.

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1. Introduction

Conventional treatment of cancer involves the selective induction of cell death through use of single or combined cytotoxic agents that target dividing tumour cells. Since most cytotoxic chemicals exhibit considerable side effects, there is continuing interest in alternative approaches that focus on selectively inducing either differentiation [1] or cellular senescence [2]. Cancer cells can undergo a form of replicative senescence termed oncogene-induced senescence (OIS), whereby the activation of oncogenic signalling pathways leads to fail-safe senescence mechanisms via a p53/p16^{INK4A}-dependent DNA damage response (DDR) [3,4]. Although OIS is often associated with tumour prevention or pre-malignant neoplasms, senescence also forms a vital part of tumour therapy, resulting from the restoration of tumour suppressors or inactivation of oncogenes to which tumours are 'addicted' [5]. Paradoxically, OIS can result from over-activation of the ERK pathway [3,6] or the AKT pathway [7,8], which under other circumstances can drive tumorigenesis. Furthermore, inhibition of the tumour suppressor phosphatase PTEN can result in a novel type of cellular senescence termed PTEN-loss-induced-senescence (PICS), which again occurs via AKT/mTOR stimulation and p53 induction [9].

Neuroblastoma (NB) is the most common and deadly extracranial solid tumour of infancy, accounting for 10% of cancers in patients under the age of 15% and 15% of deaths from this age bracket [10]. NB represents an example of a tumour that can be forced to undergo some degree of differentiation. NB cells commonly respond to retinoic acid (RA) by undergoing morphological differentiation *in vitro* [11], and this is related to the propensity of stage 4S tumours to undergo spontaneous regression *in vivo* [12]. RA treatment of minimal residual disease results in small but significant increases in patient survival [13]. However, RA resistance can evolve, leading to tumour relapse [14]. Combining RA with alternative differentiation–induction agents can nevertheless bolster its effects [15,16]. NB is therefore an appealing model for testing the efficacy of pro-differentiation and pro-senescence agents and this is perhaps made more feasible given the relative rarity of mutations in p53 [17,18] or *CDKN2A*, encoding p16^{INK4A} [19], at presentation. Indeed, senescence of NB cells occurs following the stabilization of p53 using nutlin-3, an antagonist of MDM2 [20,21]. Although RA can cause senescence in some NB cells based on their molecular profile [22], it is currently unclear whether senescence represents a significant component of the clinical response to RA.

Protein tyrosine phosphatases (PTPs) can modulate receptor-tyrosine kinase (RTK)-driven differentiation, proliferation and survival and are of interest in cancer biology [23,24]. PTP inhibition could be a reasonable strategy for triggering neuroblastoma

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differentiation, since activation of RTK-dependent AKT and ERK pathways is involved in NB cell differentiation downstream of both RA and neurotrophins [25,26]. Interestingly, broad inhibition of PTPs *in vitro* can enhance neurite outgrowth in NB cells as well as cause temporary cell cycle arrest [27,28]. We therefore hypothesised that PTP inhibitors might be effective in enhancing RA-driven cell differentiation or senescence. We assessed the effects of broad-specificity, vanadium-based PTP inhibitors in combination with RA on a number of cell lines. Not only did this indeed enhance the short-term differentiated phenotype, but a more prolonged combination treatment drove cells into a permanently differentiated or senescent state. This process is in part dependent on AKT and ERK signalling. Whereas p21 was also activated, p53 apparently was not. Thus, vanadium-based PTP inhibitors can combine with RA to enhance the stimulus for differentiation and generate a novel trigger for senescence in neuroblastoma cells.

2. Materials and methods

2.1. Cell culture

Cells were maintained in a humidified incubator at 37 °C/5% CO_2. SK-N-SH and SH-SY5Y cells were cultured in Minimum Essential Medium Eagle (Sigma–Aldrich) with 10% foetal bovine serum (FBS) and 2 mM L-glutamine. LAN-5, KELLY, SK-N-AS and SK-N-DZ human NB cells were cultured in RPMI 1640 + GlutaMAX™ (Invitrogen) with 10% FBS. SK-N-DZ, SH-SY5Y and SK-N-SH were obtained from ATCC. SK-N-AS and KELLY were provided by Frank Speleman, University of Ghent (STR genotyped). LAN-5 and SMS-KCNR were obtained from the Children's Oncology Group (COG) Cell Culture and Xenograft Repository, Texas Tech University Health Sciences Center, USA. Sodium orthovanadate (VA), N-acetyl cysteine (NAC), all-*trans* retinoic acid and VO–OH pictryhydrate were purchased from Sigma Aldrich. Bis(maltolato)oxovanadium(IV) (BMOV) was a gift from John McNeill and was also purchased from Sigma–Aldrich. U0126 was from Cell Signalling Technologies, PI103 and nutlin-3 were from Cayman Chemical Company. For NAC treatment, NAC was dissolved at 1 M in serum-free medium and the pH adjusted to approximately pH 7 with 10 M NaOH. NAC was added to cells at 20 mM for 3 h, before addition of RA and BMOV treatments.

2.2. Quantification of neurite outgrowth

Neurites were defined as processes equal to or greater than two times the length of the cell body. Neurites in single, dispersed cells were measured from the cell body to the furthest tip of the process using Openlab software (Perkin–Elmer) and the means and standard deviations of the neurite populations were calculated.

2.3. Immunocytochemistry

Cells were plated onto glass coverslips coated with poly-L-lysine (0.5 mg/ml solution in H_20) and fibronectin (10 µg/ml solution in PBS). After treatment, cells were fixed for 30 min in 4% paraformaldehyde, rinsed with PBS and pre-blocked in PBS, 1% BSA, 0.05% triton for 30 min. Anti-Ki67 antibody (Novocastra) was added in PBS, 3% BSA, 0.05% triton, followed by polyclonal goat anti-rabbit immunoglobulins/biotinylated secondary antibody (Dako), then streptavidin-conjugated cyanine-3 (GE Healthcare). Ki67-expressing cells were judged by the presence of intense nuclear foci.

2.4. Immunoblotting

Cells were lysed in 1% Triton X-100, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM proteinase inhibitor cocktail (Roche), 1 mM sodium orthovanadate and 25 mM NaF for 30 min on ice. After polyacrylamide gel electrophoresis and protein transfer to PVDF membranes, the membranes were blocked in TBS-T (50 mM Tris-base; 150 mM NaCl; 0.2% Tween-20) with 5% dried, non-fat milk (Marvel) overnight at 4 °C. Primary antibodies were incubated for 1 h, followed by HRP-conjugated secondary antibodies in TBS-T for 1 h. HRP was detected using ECLplus (Amersham Biosciences). For re-probing, blots were stripped using 0.2 M NaOH at 37 °C. Primary antibodies against the following antigens were used: pan-Trk (Santa Cruz Biotechnology); βIII tubulin (Chemicon); phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), p44/42 MAPK (ERK1/2), phospho-AKT (Ser473), AKT (Cell Signalling); p53, p21 and $p16^{INK4A}$ (Santa Cruz); β-actin (Sigma–Aldrich); PTEN (Millipore). Rabbit anti-mouse and Mouse anti-rabbit Secondary antibodies were from Dako.

2.5. Propidium iodide staining for DNA content

Cells were trypsinised, fixed in 1 ml ice-cold 70% ethanol for 30 min, rinsed twice in phosphate–citrate buffer (0.2 M Na_2HPO_4/0.1 M citric acid) by centrifugation at 2000 rpm, and resuspended in 200 µl propidium iodide solution (50 µg/ml in PBS) plus 50 µl of RNaseA solution (100 µg/ml in water). Cells were subjected to flow cytometry using a BD™ LSRII flow cytometer system (Beckman-Dickson Biosciences). A maximum of 10,000 events were collected per sample. Data was analysed using Flo-jo V8 software. G1, S, G2/M phase gates were applied to histograms by manually judging distribution peaks. Sub-G1 and polyploid cells were removed from the analysis when examining cell cycle distribution.

2.6. Senescence-associated β-galactosidase and Heterochromatin assays

Cells were seeded at low density (1×10^4 per 35 mm well) to facilitate long-term culture. Cells were fixed in 4% paraformaldehyde then rinsed with PBS and stained in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride, 150 mM sodium chloride, 30 mM citric acid/phosphate buffer (0.1 M citric acid; 0.2 M sodium phosphate dibasic; pH 6) with 1 mg/ml Xgal [29]. Cells were incubated overnight at 37 °C then photographed. For detection of heterochromatic nuclei, cells were plated on poly-L-lysine-coated coverslips and treated for 12 days with chemicals before fixing and staining with DAPI. Between 700 and 1900 cells were photographed in random fields for each treatment and the percentage of cells with heterochromatic nuclei was calculated.

3. Results

3.1. Vanadate augments retinoic acid-induced differentiation

Our initial goal was to assess the ability of vanadium-based PTP inhibitors to enhance the differentiating properties of RA in NB cells [11,27]. Three cell lines were chosen, LAN5, SK-N-SH and its derivative SH-SY5Y, as these are RA-responsive. When treated with sodium orthovanadate (VA), substantial neurite elongation was induced (Fig. 1D–F), similar to RA treatment (Fig. 1G–I). In SK-N-SH, which has a mixed population of neuronal-type (N-type) and Schwann-type (S-type) cells [30], RA induced a distinct flattening of the latter cells (arrowhead, Fig. 1I). Combined treatment with VA plus RA over 6 days resulted in significant enhancement of morphological differentiation, with elaborate neurite networks harbouring neuronal-like and fusiform cells (Fig. 1J–L). Combined VA/RA treatment was accompanied by gains in average neurite length in SK-N-SH and SH-SY5Y, compared to single treatments (Fig. 1M). Furthermore, the percentages of cells bearing neurites increased additively with the VA and RA combination (Fig. 1M). Under the 6-day combined treatment, the SK-N-SH S-type cells gained a highly fusiform phenotype. Alongside the changes in morphology, VA enhanced the RA-mediated upregulation of Trk proteins in SH-SY5Y and LAN-5 cells, as well as increasing βIII-tubulin in SH-SY5Y (Fig. 1N). A second vanadium derivative, the organometallic bis(maltolato)oxovanadium(IV) (BMOV), has previously proved to be less toxic and more effective than inorganic vanadium salts in regulating insulin resistance in rodent models of diabetes [31,32]. In the present study, BMOV also proved to be very effective at inducing neuritogenesis (Supplementary Fig. 1). Combination treatment for 3–6 days therefore generated greater differentiation in response in NB cells, compared to RA alone or vanadium compounds alone.

Although RA induces differentiation in numerous NB cell lines, it is not always clear how effectively the cells have been forced out of the cell cycle. Using timelapse microscopy we still observed dividing cells after 6 days of the above treatments (data not shown). We therefore examined both the expression pattern of the cell cycle marker Ki67 (Fig. 2A), as well as the proportion of cells in each stage of the cycle (Fig. 2B). Although RA induced 50–80% reductions in Ki67-labelled cells, there remained many cells in cycle. Flow cytometry revealed that in SH-SY5Y and LAN-5, RA generated a modest accumulation in G0/G1 and depletion of S-phase cells, as well as a depletion of G2-phase cells in

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