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TP-0903 inhibits neuroblastoma cell growth and enhances the sensitivity to conventional chemotherapy

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ABSTRACT

Neuroblastoma (NB) is an embryonal tumor with low cure rate for patients classified as high-risk. This class of NB tumors shows a very complex genomic background and requires aggressive treatment strategies. In this work we evaluated the efficacy of the novel multi-kinase inhibitor TP-0903 in impairing NB cells' growth, proliferation and motility. *In vitro* studies were performed using cell lines with different molecular background, and *in vivo* studies were done using the zebrafish experimental model.

Our results confirmed a strong cytotoxicity of TP-0903 already at the sub-micro molar concentrations. The observed cytotoxicity of TP-0903 was irreversible and the resulting apoptosis was caspase dependent. In addition, TP-0903 impaired colony formation and neurosphere creation. Depending on the molecular background of the selected NB cell lines, TP-0903 influenced either their capacity to migrate, to complete their cell cycle or both. Likewise, TP-0903 reduced NB cells intravasation *in vitro* and *in vivo*. Importantly, TP-0903 showed remarkable pharmacological efficacy not only as a mono-treatment, but also in combination with conventional chemotherapy drugs (ATRA, cisplatin, and VP16) in different types of NB cells.

In conclusion, the multi-kinase activity of TP-0903 allowed the impairment of several biological processes required for expansion of NB cells, making them more vulnerable to the conventional chemotherapeutics. Altogether, our results support the eligibility of TP-0903 for further (pre)clinical assessments in NB.

1. Introduction

Neuroblastoma (NB) is a tumor of the sympathetic nervous system that occurs predominantly in infants, toddlers and children at pre-scholar age (Luksch et al., 2016; Maris et al., 2007). Although the effectiveness of therapy settings for pediatric cancers is constantly improving, a portfolio of drugs regarding patients with high-risk (HR) NB has not been significantly amended yet. Therefore, the introduction of novel compounds is necessary (Lonergan et al., 2003). One of the reasons for poor survival of HR-NB patients is the elevated biological variability among the NB tumors (Brodeur, 2003). Analysis of the entire genome of patients with NB, by the next-generation sequencing, revealed a very large intra-tumoral heterogeneity (Esposito et al., 2017; Pugh et al., 2013). Beside well known prognostic factors for NB, such as *ALK, MYCN* and *PHOX2B* (Raabe et al., 2008), the functional analyses that followed the whole-genome sequencing confirmed the relevance of additional molecules that might determine future clinical improvements (Peifer et al., 2015; Zage et al., 2012) or unravel new insights about already known targets (Bellini et al., 2015).

Protein kinases are well known molecular markers in a variety of malignancies, and their targeting represents a very promising treatment strategy. The TAM protein family of receptor tyrosine kinases, particularly AXL, was proposed to be of particular relevance in conferring survival advantages to cancer cells (Graham et al., 2014). For these reasons, a raising interest of biologists and oncologists is directed toward AXL targeting (Verma et al., 2011). AXL is an important tumor propagator that impacts cell migration, adhesion and response to drugs through several signaling pathways: the extra-cellular signal-regulated kinase (ERK), the phosphatidylinositol 3-kinase/AKT and GTP-ases from Rho protein family (Korshunov, 2012). Also, the involvement of AXL oncoprotein in NB pathology has been reported recently (Debruyne et al., 2015). Considering that all beforehand mentioned AXL-

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dependent pathways were found deregulated in NB (Brodeur, 2003), the inhibition of AXL, as their governor, would theoretically abolish the survival advantages that they assure to NB cells.

Another clinically relevant kinase for NB tumors is Aurora A (AURKA). While the precise role of AXL in pathology of NB tumors is currently under investigation, AURKA represents a clear negative prognostic marker for NB patients (Ramani et al., 2015). Beside the regulation of the cell cycle and progression of mitosis, AURKA also protects MYCN from degradation (Otto et al., 2009). These attributes made AURKA an attractive target for decades (Romain et al., 2014), and a search for a more effective AURKA inhibitor is still ongoing.

Lately, a new multi-kinase drug called TP-0903, able to inhibit either AXL or AURKA, has been proposed (Myers et al., 2016). TP-0903 was highly efficient in the treatment of chronic lymphocytic leukemia and invasive breast cancer (Sinha et al., 2015; Soh et al., 2016). Its successful activity against other types of cancer prompted us to evaluate whether TP-0903 could confirm the same anti-tumoral effects in NB cells as well. We established the tumor cell directed cytotoxicity which caused an irreversible impairment of NB cells growth, sustaining the TP-0903 for further (pre)clinical evaluations in this cancer.

2. Materials and methods

2.1. Cell culturing and treatments

The human NB tumor cell lines NB3, kindly provided by Dr Luca Longo (IRCCS AOU San Martino-IST, Genoa), SH-SY5Y (from DSMZ) and IMR32 (from ATCC), were cultured in RPMI medium (Sigma-Aldrich, Milan, Italy). The selectivity of TP-0903 towards transformed cells and not normal ones was tested using Hek293T and BJ fibroblast cells. In the lack of healthy human neuroblasts as non-tumoral control for our study, we relied on the findings of Shaw et al. (Shaw et al., 2002) that attributed certain neuro-specific features to Hek293T cell line, which was kindly provided by Dr Stefano Indraccolo (IOV, Padua). The BJ cell line was kindly provided by Dr Michela Pozzobon (IRP CDS, Padua). Both non-cancer, control cell lines were grown in DMEM medium enriched with antibiotics and glutamine (1%), fetal bovine serum (FBS; 10%; all from Gibco, Life Technologies, Monza, Italy). Cell lines authentication was done at the BMR Genomics, Padua. Cell culture media were tested by PCR to exclude mycoplasma infections. The primary human umbilical vein endothelial cells (HUVEC) were purchased from Life Technology and maintained in M200 medium with addition of Low Serum Growth Supplement (LSGS; Life technology) until the sixth passage.

All treatments were done using less than 0.1% of DMSO (Sigma-Aldrich) as control and the following chemical agents: all-trans retinoic acid (ATRA), VP16, Cisplatin, SB203580, Z-VAD, 5-aza-2′deoxycytidine (AZA; all from Sigma-Aldrich) and TP-0903 (Selleck Chemicals, Munich, Germany). Concentrations used for single or combined experiments are mentioned within the text.

2.2. Viability assay and percent growth calculation

Cells viability was determined 24 h, 48 h and 72 h post-treatment by measuring the amount of the resulting formazan, using Multilabel Plate Reader VICTOR (PerkinElmer, Waltham, MA) at 486 nm, and the number of cells used for the analyses was determined as described in detail before (Aveic et al., 2016). Results were calculated with respect to the starting conditions or to the DMSO control treatments. Growth rate percentage was used to quantify the change over time in treated and control samples respect to the initial conditions (0 h). Similarly, the MTT assay served for the Inhibitory Concentration 50% (IC_{50}) determination.

2.3. Cell growth recovery measurement

The capacity of cells to recover from TP-0903 treatment was measured after drug wash-out. Cells were plated as described in detail elsewhere (Aveic et al., 2016), and treated 24 h with increasing concentrations of TP-0903 (10, 20, 40, 80 and 100 nM) and DMSO. Afterwards, cell medium was removed and substituted with fresh drug-free RPMI. Cell viability was controlled after 24 h (24 h with TP-0903 + 24 h without TP-0903) and 48 h (24 h with TP-0903 + 48 h without TP-0903), and changes in cell growth were calculated as percent growth relative to the starting conditions applying the MTT assay.

2.4. Determination of cells' proliferation rate

The 5-ethynyl-2'-deoxyuridine (EDU)–Click kit (BCK-Edu488, Sigma-Aldrich) was used for the evaluation of DNA synthesis as suggested by manufacturer. Shortly, 1×10^5 NB cells were seeded in the chamber slides one day before the treatment. Then TP-0903 was left for 24 h adding the EdU solution (10 μM) for 8 h before fixation with 3.7% formaldehyde (Sigma-Aldrich). After permeabilization with 0.5% Triton-X100, the reaction mix was added and the experiment carried out as recommended by the protocol. EdU-positive cells (green) were counted under the inverted fluorescence microscope (Vico, Eclipse Ti80, Nikon, Tokyo) and normalized to the number of total nuclei (stained with DAPI; blue).

2.5. RNA isolation, cDNA synthesis and quantitative RT-PCR analysis (qPCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, Life Technologies) and cDNA synthesized using Super Script II (Invitrogen), according to the manufacturer's recommendations. One-twentieth of produced cDNA was then used to determine gene expression by the ABI prism 7900 (Applied Biosystems, Forest City, CA) using the SYBR Green PCR Master Mix (Applied Biosystems) as described before (Aveic et al., 2016). The primer sequences are available upon request.

2.6. DNA isolation, bisulfite conversion and sequencing

Genomic DNA from SH-SY5Y and IMR32 was extracted using columns (Invisorb Spin Tissue Mini Kit, Stratec), and 1 μg was treated with sodium bisulfite (EpiTect Bisulfite kit; Qiagen) according to the manufacturer's instructions. Bisulfite converted DNA was subjected to PCR reaction using High fidelity Taq polymerase (Qiagen) to amplify zone of CpG island found within the *AXL* gene promoter. Primers for the reaction were designed with the MethPrimer program: *AXLbisu*F 5'-GTTTGAGTGTGTTTGTGGGTTAGTA-3', *AXLbisu*R 5'-CAAACCTCCTTA ACCCTTCATT-3' (Li and Dahiya, 2002); the PCR amplicons were cloned in TOP10 competent bacteria (Invitrogen) following manufacturer instructions. Ten colonies per sample were sequenced using BigDye Terminator Cycle Sequencing Kit v3.1 on the ABI PRISM 3500DX Genetic Analyzer sequencer (Applied Biosystems). The same sequencing procedure was used to check for the presence of exon 10 in *AXL* mRNA. DNA fragmentation was validated on 1% agarose gel (Invitrogen), after being processed as described elsewhere (Kasibhatla et al., 2006).

2.7. Wound healing (scratch) and Boyden chamber assays

Details for wound healing assay (IBIDI, Milano, Italy) settings were described previously (Aveic et al., 2016). Wound healing was analyzed at 0 h, 24 h and 48 h by measuring the percentage of scratched area (ImageJ software; NIH, Bethesda, MD). To avoid that the cell proliferation influence on the final assay results, the cells were kept under low serum (3% FBS) conditions. For cell migration measurements, the cells in DMSO or TP-0903 were plated on 8 μm porous membrane (BD