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Targeted *MYCN* expression affects cytotoxic potential of chemotherapeutic drugs in neuroblastoma cells

Tobias Paffhausen, Manfred Schwab, Frank Westermann *

Division of Tumour Genetics, German Cancer Research Center, DKFZ, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

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Abstract

Neuroblastoma (NB) is a solid childhood tumour that exhibits heterogeneous biological and clinical phenotypes. Multiple drug resistance marks a major complication especially in high-risk patients with advanced tumour stages and specific genetic aberrations, such as *MYCN* amplification and lp deletion. As an approach to further address the mechanisms of chemotherapeutic responsiveness of NB, we used a *MYCN*-inducible *in vitro* system and tested the susceptibility of NB cells to anti-tumour drugs currently included in NB treatment protocols dependent on *MYCN* expression. We observed cytotoxic effects using drug concentrations corresponding to blood plasma levels achieved in NB patients. The most potent drugs were microtubule inhibitors vindesin, paclitaxel and vincristin. Less effective were doxorubicine, arsenic trioxide, cisplatin, etoposide and carboplatin. Exposed to anti-tumour agents, NB cells with induced *MYCN* expression exhibited higher specific apoptosis than NB cells lacking *MYCN* expression. Anti-tumour drugs in *MYCN*-on cells accelerated G1–S phase transition, led to enhanced accumulation of cell populations in G2/M phase, and increased levels of apoptosis. In contrast, *MYCN*-off cell populations arrested in G1 and, to a smaller extent, in G2/M and exhibited delayed onset of apoptosis. In summary, apoptosis profiles and anti-proliferative potential of chemotherapeutic drugs, used at *in vivo* tolerable doses, are affected by *MYCN* overexpression and deregulated cell cycle in SH-EP^{MYCN} cells.

Keywords: Chemotherapeutic drugs; *MYCN*; Apoptosis; Cell cycle; Cytotoxicity; Neuroblastoma

1. Introduction

NB is the second most common solid cancer in young children accounting for 9% of all childhood cancers [1]. It is characterised by a heterogeneous clinical behaviour that ranges from spontaneous regression in 10% of all cases to rapid and largely treatment resistant progression with fatal outcome. At present, German patient risk classification includes *MYCN* gene amplification, 1p chromosomal deletion, tumour stage, and age of the child at diagnosis [2–4]. Amplification of the *MYCN* oncogene [5] is present in about 25–30% of primary untreated NBs, which is associated with advanced stage disease, rapid progression and unfavourable prognosis [6]. Moreover, this patient subgroup often demonstrates a multiple drug resistant (MDR) phenotype that develops from exposure to chemotherapeutic agents and increases with intensity of the therapy accommodated [7].

* Corresponding author. Tel.: +49 6221 42 3275; fax: +49 6221 42 3277.
E-mail address: f.westermann@dkfz.de (F. Westermann).

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NB treatment includes induction chemotherapy, maintenance of high dose chemotherapy, radiotherapy, tumour surgery and consolidation therapy [8]. Chemotherapeutic protocols combine alkylating agents with microtubule active drugs, topoisomerase inhibitors and antibiotics. Anti-neoplastic drugs (12–15) are effectively used in the treatment of NB patients [9]. Following initial treatment with cytotoxic drugs, NB tumours are highly chemoresponsive, displaying significant partial or complete remission in about 80% of tumours, even those with unfavourable prognostic outcome [7]. Enhanced *MYCN* expression at least partly accounts for these initial regression as targeted *MYCN* sensitises NB cells for doxorubicine-induced apoptosis *in vitro* [10]. Cooperation of overexpressed *MYCN*, consequent to amplification, together with deregulated cell death pathways may lead to a chemoresistant phenotype and adverse progression of the disease *in vivo*. Despite intensive chemotherapy, 30% 5-year event-free survival and 15–20% long-term event-free survival are still unsatisfactory in this high risk patient subgroup. Hence, better understanding of drug response mechanisms using *in vitro* and *in vivo* models hold the promise of improving chemotherapy for NB patients.

In this study, a well-established NB model was applied [11], which harbours a doxycycline-controlled *MYCN* gene expression system. It was previously shown that targeted *MYCN* expression enhances the rate of DNA synthesis and cell proliferation, shortens G1–S phase transition and sensitises NB cells for drug and cytokine-induced apoptosis [12]. Thus the human NB cell line SH-EP^{MYCN}, serves as a suitable *in vitro* model to investigate molecular processes of drug-induced cell death. Here, our aim was to examine the impact of various anti-tumour drugs on cell survival within this NB *in vitro* system. Cells with induced or uninduced *MYCN* were exposed to different drugs. Specific apoptosis was determined by fluorescence-activated cell sorting (FACS) analysis and drug response profiles were established. Vincristin, vindesin, doxorubicine, etoposide, cisplatin, melphalan, carboplatin and dacarbacine, which are currently administered to NB patients, were tested according to *in vivo* tolerable doses. Paclitaxel as a mitotic inhibitor and betulinic acid and arsenic trioxide as mitochondrial effectors were also included, although these are presently not applied during NB treatment. We observed different cytotoxicities of chemotherapeutic agents and altered cell cycle distribution, which were both dependent on the *MYCN* expression in SH-EP^{MYCN} cells.

2. Materials and methods

2.1. Cell culture and drug treatment

The SH-EP cell line Tet-21N (SH-EP^{MYCN}) was cultured in RPMI 1640 medium supplemented with 10% or 0.5% (v/v) FBS and penicillin (100 IU/ml)/streptomycin (100 μg/ml). Cultures were maintained at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. Cells were plated at a density of 3×10^4 per well in 12-well plates. Induction of *MYCN* expression was regulated by removal of doxycycline (l00 ng/ml). Cells were treated for 24 and 48 h with cytotoxic drugs at concentrations in the range of 2–4 logs corresponding to therapeutic doses in NB patients (Table 1). Arsenic trioxide (As_2O_3), doxorubicine (DOX), etoposide (VP-16), melphalan (MEL) and vindesin (VDS) were purchased from Sigma–Aldrich, betulinic acid (BetA), paclitaxel (PTX) and vincristine (VCR) were obtained from Alexis Biochemicals, carboplatin (CBP), cisplatin (CDDP) and dacarbacine (DTIC) came from LKT laboratories.

2.2. Determination of apoptosis and cell cycle distribution

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified essentially as described previously [13]. Briefly, 3×10^4 cells were seeded in 1.5 ml medium in 12-well plates (Corning Inc., NY) as triplets. Cells were incubated with or without cytostatic compounds for indicated times and given drug concentrations (Table 1) resembling clinical tolerable doses. Control samples were used properly corresponding to the concentration of solvent present at the respective dilution of chemotherapeutic drugs. Samples were collected by centrifugation at 800*g* for 5 min, washed once with PBS and resuspended in 200 μl of lysis solution containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium citrate, and 50 μg/ml propidium iodide (PI). After incubation at 4 °C in the dark over night, apoptosis and cell cycle distribution were quantitatively determined by FACS analysis (FACScan cytometer Becton–Dickinson, San Jose, CA) of PI stained nuclei. Ten thousand events per sample were measured. Specific apoptosis was determined by subtraction of the control values. Data are expressed as means ± SD; $n \geqslant 3$.

3. Results

3.1. Vindesin, paclitaxel and vincristin most effectively induce apoptotic cell death

We used the neuroblastoma *in vitro* system SH-EP^{MYCN} to analyse the impact of chemotherapeutic drugs on cell proliferation and apoptosis. Drug-induced cell