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Targeted BIRC5 silencing using YM155 causes cell death in neuroblastoma cells with low ABCB1 expression

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ABSTRACT

The BIRC5 (Survivin) gene is located at chromosome 17q in the region that is frequently gained in high risk neuroblastoma. BIRC5 is strongly over expressed in neuroblastoma tumour samples, which correlates to a poor prognosis. We recently validated BIRC5 as a potential therapeutic target by showing that targeted knock down with shRNA's triggers an apoptotic response through mitotic catastrophe. We now tested YM155, a novel small molecule selective BIRC5 suppressant that is currently in phase I/II clinical trials. Drug response curves showed IC50 values in the low nM range (median: 35 nM, range: 0.5–>10,000 nM) in a panel of 23 neuroblastoma cell lines and four TIC-lines, which resulted from an apoptotic response. Nine out of 23 cell lines were relatively resistant to YM155 with IC50 values >200 nM, although in the same cells shRNA mediated knock down of BIRC5 caused massive apoptosis. Analysis of differentially expressed genes between five most sensitive and five most resistant cell lines using Affymetrix mRNA expression data revealed ABCB1 (MDR1) as the most predictive gene for resistance to YM155. Inhibition of the multi-drug resistance pump ABCB1 with cyclosporine or knockdown with shRNA prior to treatment with YM155 demonstrated that cell lines with ABCB1 expression became 27–695 times more sensitive to YM155 treatment.

We conclude that most neuroblastoma cell lines are sensitive to YM155 in the low nM range and that resistant cells can be sensitised by ABCB1 inhibitors. Therefore YM155 is a promising novel compound for treatment of neuroblastoma with low ABCB1 expression.

1. Introduction

BIRC5 (Survivin) is an inhibitor of apoptosis protein (IAP) with a crucial function in cell cycle and apoptotic signalling. In the intrinsic apoptotic pathway it can bind and inhibit the pro-apoptotic protein DIABLO and it can bind and stabilise XIAP, another IAP. Inhibition of this function of BIRC5 induces apoptosis by activating the intrinsic apoptotic pathway.[1–3] In addition, BIRC5 can stabilise microtubules in the chromosomal passenger complex during mitosis. Inactivation of BIRC5 can therefore also lead to mitotic catastrophe which activates the intrinsic apoptotic pathway via p53 and Caspase 2.[3–7] Genomic aberrations of the BIRC5 locus at 17q occur in several malignancies. BIRC5 is gained in almost all high risk neuroblastoma which is a paediatric tumour that originates from the neural crest derived precursor cells of the sympathetic nervous system.[8–10] BIRC5 over expression in these tumours strongly correlates to a poor prognosis. BIRC5

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knockdown in neuroblastoma causes apoptosis via mitotic catastrophe, suggesting that in these tumour cells the crucial function of BIRC5 is microtubule stabilisation.[11]

In addition to gain of the BIRC5 locus at 17q, only few other aberrations in apoptotic signalling have been reported in neuroblastoma tumours and cell lines.[12,13] P53 mutations are rare and many cell line experiments showed that p53 can be activated to induce apoptosis.[14,15] Caspase 8 is hypermethylated and thereby inactive in some neuroblastoma resulting in an inactive extrinsic apoptotic pathway.[16] And finally BCL2 is often over expressed in neuroblastoma tumours and has been found to be a target for therapy.[17,18]

Thus, BIRC5 is one of the few drugged targets in the intrinsic apoptotic pathway. This warrants further validation in neuroblastoma since current treatment regimens can only cure 25–35% of high stage neuroblastoma patients and there is a strong need for new targeted therapies.[8–10] BIRC5 has shown to be a viable therapeutic target and several new strategies for inhibiting BIRC5 have recently become available. The locked nucleic acid (LNA)[19] based antisense molecule EZN3042 was effective *in vitro* in NB cells.[11] The anti-BIRC5 antisense LNA oligonucleotide LY2181308 (gataparsen sodium) is currently being tested in phase II clinical trials in solid tumours and BIRC5 based vaccines are currently in phase I/II clinical trial.[20] Though targeted therapy by antisense based compounds can be effective in haematological malignancies, they have been disappointing in solid tumours. A promising new small molecule BIRC5 suppressant is YM155, developed by Astellas Pharma. This compound was selected by high throughput screening with a BIRC5 Promoter Luciferase Assay and inhibits mRNA expression of BIRC5.[21] Phase I/II clinical 'single agent' trials showed acceptable toxicity in patients with advanced solid malignancies.[22,23] In a phase II trial in melanoma the pre-specified criterion for success was not reached,[24] but in non-small-cell-lung-cancer 5% of the patients showed a partial response, and 38% showed stable disease.[25] YM155 has also induced responses in a phase I trial in patients with non-Hodgkin's lymphoma or prostate cancer.[23]

In this paper we investigated the efficacy of YM155 in 23 neuroblastoma cell lines and four neuroblastoma 'tumour initiating cell' (TIC) lines. First, we validated BIRC5 as a therapeutic target by lentiviral shRNA mediated silencing of BIRC5, which resulted in massive apoptosis in all six neuroblastoma cell lines tested. Subsequent assays using YM155 induced apoptosis in the majority of 23 tested neuroblastoma cell lines as well. Surprisingly, some cell lines that were sensitive for targeted silencing using BIRC5 shRNA were resistant to YM155. Analysis of mRNA profiles of sensitive and insensitive cell lines identified the multi-drug resistance pump ABCB1 (MDR1),[26–28] as the best predictor of resistance. Inhibition of ABCB1 with cyclosporine or lentiviral shRNA sensitised the resistant cell lines to YM155 induced apoptosis.

2. Methods

2.1. Cell lines

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum, 10 mM L-glutamine, 10 U/ml penicillin/streptomycin, non-essential amino acids (1×) and 10 μg/ml streptomycin. Cells were maintained at 37 °C under 5% CO_2. For primary references of these cell lines, see Molenaar et al.[29] The tumour initiating cell (TIC) lines were isolated directly from patient tumour or bone marrow cells and cultured in neural specific stem cell medium (400 ml DMEM glutamax, 133 ml F12 medium, 2% B27, 20 ng/ml EGF, 40 ng/ml FGF, 10 U/ml penicillin/streptomycin) as described previously.[30]

2.2. Lentiviral shRNA production and transduction

Lentiviral particles were produced in HEK293T cells by cotransfection of lentiviral vector containing the short hairpin RNA (shRNA) with lentiviral packaging plasmids pMD2G, pRRE and pRSV/REV using FuGene HD. Supernatant of the HEK293T cells was harvested at 48 and 72 h after transfection, which was purified by filtration and ultracentrifuging. The concentration was determined by a p24 ELISA.

Cells were plated in a 10% confluence. After 24 h cells were transduced with lentiviral BIRC5 shRNA (Sigma, TRCN0000073720; coordinates: chromosome 17; 76212781–76212801; hg19), or ABCB1 shRNA B5 and B7 (Sigma, TRCN0000059684; coordinates: chromosome 7; 87190611–87190631; hg19, and TRCN0000059686; coordinates: chromosome 7; 87175290–87175310; hg19) in various concentrations (multiplicity of infection (MOI): 1–3). SHC-002 shRNA (non-targeting shRNA: CAACAAGATGAAGAGCACCAA) was used as a negative control. Twenty-four hours after transduction medium was refreshed and puromycin was added to determine the efficacy of transduction. Protein was harvested 72 h after transduction and analysed by Western blot. Nuclei were harvested 48 and 72 h after transfection for FACS analysis.

2.3. Lentiviral over expression clones

BIRC5 over expression constructs 7 and 10 were produced from a PCR product of BIRC5 (CCDS11755.1: isoform 1) that was obtained from IMR32 cDNA (primers: TATATAGGATCCATTAACCGCCAGATTTGA/TATATAGAATTCGGTGGCACCAGGGAATAAAC) and cloned into pLenti4/TO/V5-Dest according to the manufacturer's procedures (Invitrogen). The sequence has been checked using the manufacturer's primers (pL4-TO/V5 fwd and pL4-dest rev).

2.4. Compounds

YM155 (provided by Astellas Pharma) was dissolved in DMSO in a stock concentration of 10 mM. It was added to the cells in concentrations from 0.1 nM to 10 μM 24 h after plating the cells in 10–30% confluence. Cyclosporine (Sigma, C3662) was added to the cells in a concentration of 5 μM, 24 h after plating. The cells were incubated with cyclosporine for 1 h before YM155 was added without removal of cyclosporine.

RNA extraction and Affymetrix profiling, MTT-assay, Western blotting and antibodies, FACS analysis and Crystal Violet assays are described in the Supplementary data.