New therapeutic targets in the intrinsic apoptotic pathway in neuroblastoma
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Targeted BIRC5 Silencing Using YM155 Causes Cell Death in Neuroblastoma Cells with Low ABCB1 Expression
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 tumor 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 nM->10,000 nM) in a panel of 23 neuroblastoma cell lines and four TIC-lines, which resulted from an apoptotic response. Nine out 23 cell lines were relatively resistant to YM155 with IC50 values >200nM, although in the same cells shRNA mediated knock down of BIRC5 caused massive apoptosis. Analysis of differentially expressed genes between 5 most sensitive and 5 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 to 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 sensitized by ABCB1 inhibitors. Therefore YM155 is a promising novel compound for treatment of neuroblastoma with low ABCB1 expression.
Introduction

BIRC5 (Survivin) is an Inhibitor of Apoptosis Protein (IAP) with a crucial function in cell cycle and apoptotic signaling. In the intrinsic apoptotic pathway it can bind and inhibit the pro-apoptotic protein DIABLO and it can bind and stabilize XIAP, another IAP. Inhibition of this function of BIRC5 induces apoptosis by activating the intrinsic apoptotic pathway.\(^1-3\) In addition, BIRC5 can stabilize 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 TP53 and Caspase 2.\(^3-7\) Genomic aberrations of the \textit{BIRC5} locus at 17q occur in several malignancies. \textit{BIRC5} is gained in almost all high risk neuroblastoma which is a pediatric tumor that originates from the neural crest derived precursor cells of the sympathetic nervous system.\(^8-10\) \textit{BIRC5} over expression in these tumors strongly correlates to a poor prognosis. BIRC5 knockdown in neuroblastoma causes apoptosis via mitotic catastrophe, suggesting that in these tumor cells the crucial function of BIRC5 is microtubule stabilization.\(^11\)

In addition to gain of the \textit{BIRC5} locus at 17q, only few other aberrations in apoptotic signaling have been reported in neuroblastoma tumors and cell lines.\(^12,13\) \textit{TP53} mutations are rare and many cell line experiments showed that TP53 can be activated to induce apoptosis\(^14,15\). \textit{Caspase 8} is hypermethylated and thereby inactive in some neuroblastoma resulting in an inactive extrinsic apoptotic pathway.\(^16\) And finally \textit{BCL2} is often over expressed in neuroblastoma tumors 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 tumors and BIRC5 based vaccines are currently in Phase I/II clinical trial.\(^20\) Though targeted therapy by antisense based compounds can be effective in hematological malignancies, they have been disappointing in solid tumors. 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\textsuperscript{21}. Phase I/II clinical ‘single agent’ trials showed acceptable toxicity in patients with advanced solid malignancies.\textsuperscript{22,23} In a Phase II trial in melanoma the pre-specified criterion for success was not reached\textsuperscript{24}, but in Non-small-cell-lung-cancer 5\% of the patients showed a partial response, and 38\% showed stable disease\textsuperscript{25}. YM155 has also induced responses in a phase I trial in patients with non-Hodgkin’s Lymphoma or prostate cancer.\textsuperscript{23}

In this paper we investigated the efficacy of YM155 in 23 neuroblastoma cell lines and 4 neuroblastoma ‘Tumor 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 6 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 \textit{ABCB1} (MDR1)\textsuperscript{26-28}, as the best predictor of resistance. Inhibition of ABCB1 with cyclosporine or lentiviral shRNA sensitized the resistant cell lines to YM155 induced apoptosis.

\textbf{Methods}

\textit{Cell lines}

All cell lines were grown in Dulbecco Modified Eagle Medium (DMEM), supplemented with 10\% fetal calf serum, 10 mM L-glutamine, 10 U/ml penicillin/streptomycin, Non Essential Amino Acids (1x) and 10 μg/ml streptomycin. Cells were maintained at 37 °C under 5\% CO\textsubscript{2}. For primary references of these cell lines, see Molenaar et al\textsuperscript{29}. The Tumor Initiating Cell (TIC) lines were isolated directly from patient tumor or bone marrow cells and cultured in neural specific stem cell medium (400 ml DMEM glutamax, 133 ml F12 medium, 2\% B27, 20ng/ml EGF, 40 ng/ml FGF, 10 U/ml penicillin/streptomycin) as described previously\textsuperscript{30}.

\textit{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 HET293T
cells was harvested at 48 and 72 hours 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 hours 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: CAACAAGATGAAGAGACCAA) was used as a negative control. 24 hours after transduction medium was refreshed and puromycin was added to determine the efficacy of transduction. Protein was harvested 72 hours after transduction and analyzed by Western blot. Nuclei were harvested 48 and 72 hours after transfection for FACS analysis.

**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: TATATAGATCCATTAACCGCCAGATTTGA/TATATAGAATTCGGTGGCACCAGGGAATAAAC) and cloned into pLenti4/TO/V5-Dest according to manufacturer’s procedures (Invitrogen). The sequence has been checked using the manufacturer’s primers (pL4-TO/V5 fwd and pL4-dest rev)

**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 hours after plating the cells in 10 to 30% confluence. Cyclosporine (Sigma, C3662) was added to the cells in a concentration of 5 µM, 24 hours after plating. The cells were incubated with cyclosporine for 1 hour before YM155 was added without removal of cyclosporine.

**RNA extraction and Affymetrix profiling**

For profiling total RNA of neuroblastoma cell lines was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. RNA concentration was determined using the NanoDrop ND-1000 and quality was determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). For Affymetrix Microarray analysis, fragmentation of RNA, labeling, hybridization to HG-U133 Plus 2.0 microarrays and scanning was carried out according to the
manu facturer’s protocol (Affymetrix Inc.). The expression data were normalized with
the MAS5.0 algorithm within the GCOS program of Affymetrix. Target intensity was
set to 100 (\(\alpha_1=0.04\) and \(\alpha_2=0.06\)). If more than one probe set was available for one
gene the probe set with the highest expression and most present calls was selected,
considered that the probe set was correctly located on the gene of interest. Mostly
this is a probe set at the 3’ end. The data were analyzed with the R2 microarray
analysis and visualization platform (http://r2.amc.nl).

**MTT-assay**

Forty-eight hours after treatment with YM155, 10 µl of Thyazolyl blue tetrazolium
bromide (MTT, Sigma M2128) was added to the cells. After 4-6 hours of incubation
100 µl of 10% SDS, 0.01 M HCl was added to stop the reaction. The absorbance
was measured at 570 nm and 720 nm using a platereader (biotek). The IC50
(concentration drug needed for 50% cell viability reduction) was calculated using
concentration vector curves.

**Western Blotting**

Twenty-four to forty-eight hours after treatment with YM155 or 48 – 72 hours after
transduction with shRNA, attached and floating cells were harvested on ice. Cells
were lysated with Laemmli buffer (20% glycerol, 4% SDS, 100mM Tris HCl pH 6.8
in mQ). Protein was quantified with RC-DC protein assay (Bio-Rad). Lysates were
separated on a 10 % SDS-Page gel and electroblotted on a transfer membrane
(Millipore, IPFL00010). Blocking and incubation were performed in OBB according
to manufacturer’s protocol (LI-COR). Primary antibodies used were BIRC5 (rabbit
monoclonal antibody, cell signaling: 2808), PARP (rabbit polyclonal antibody, cell
signaling: 9542), ABCB1 mouse monoclonal (abcam, ab3364) and β-actin mouse
monoclonal (abcam, ab6276). The secondary antibodies used were provided by LI-
COR. Proteins were visualized with the Odyssey bioanalyzer (LI-COR) and protein
expression was quantified with the Odyssey software.

**FACS analysis**

Seventy-two hours after treatment with YM155 both the attached and the floating
cells were fixed with 100% ethanol at -20 ºC. After fixing, the cells were stained
with 0.05 mg/ml propidium iodide and 0.05 mg/ml RNAse A in PBS. After 1 hour
incubation, DNA content of the nuclei was analyzed using a fluorescence activated
cell sorter (Accuri). A total of 10,000 nuclei per sample were counted. The cell cycle
distribution and apoptotic sub G1 fraction was determined using Flowjo.
Crystal Violet
Forty-eight hours after treatment the cells were fixed with 100% ice cold methanol for 10 minutes and stained with Crystal Violet (0.5% Crystal Violet in 25% MeOH/ 75% ddH2O) for 10 minutes. Wells were rinsed with ddH2O.

Results

BIRC5 shRNA induces apoptosis in neuroblastoma cell lines
We first validated BIRC5 as a drug target by silencing the expression using shRNA targeting the coding sequence of BIRC5 in a series of neuroblastoma cell lines. This resulted in a massive phenotypic response 72 hours after transduction in all six tested neuroblastoma cell lines (fig 1a). Western blot analysis confirmed targeted knockdown of BIRC5 in all cell lines tested. BIRC5 silencing resulted in PARP cleavage which confirms that the cells die from an apoptotic response (fig 1b). These findings establish targeted silencing of BIRC5 as a potential therapeutic intervention in neuroblastoma tumor cells and we therefore decided to test the efficacy of the small molecule BIRC5 suppressant YM155 in neuroblastoma cell lines.

YM155 sensitivity in neuroblastoma cell lines
First we determined the IC50 (concentration drug needed for 50% cell survival) for a panel of 23 neuroblastoma cell lines using an MTT-assay. This showed that 14 out of 23 cell lines were sensitive to YM155, with an IC50 below 200 nM (table 1). Examples of sensitive cell lines are SKNAS, IMR32 and SMSKCNR of which the dose-effect curves show sensitivity to YM155 in the low nM range (fig 2a). Pictures of SKNAS and IMR32 illustrate the increasing phenotypic response after treatment with accruing concentrations of YM155 (suppl fig 1a) and Crystal Violet assays showed a clear decrease of attached cells in SMSKCNR and SKNAS (suppl fig 1b). In addition to classical cell lines, we tested the sensitivity of 4 newly isolated TIC-lines to YM155. These cells were isolated directly from patient tumor or bone marrow material and maintained in neural stem cell specific medium. These TIC lines also showed sensitivity to YM155 in the low nM range (table 1).

YM155 causes apoptosis by specific silencing of BIRC5
To evaluate the phenotype after treatment with YM155, we performed Western blot analysis. SKNAS and IMR32 showed dose-dependent BIRC5 silencing, PARP cleavage occurred at 50 nM for SKNAS and 10 nM for IMR32 (fig 2b). FACS analysis
of SKNAS, IMR32 and SMSKCNR showed a large increase of the sub-G1 fraction of 32, 20 and 10 fold respectively, 48 hours after treatment with 10 nM YM155 (fig 2c). These findings indicate that YM155 causes targeted silencing of BIRC5, which induces apoptosis.

To verify if the apoptotic effect of YM155 is caused specifically by BIRC5 inhibition, we induced ectopic BIRC5 over expression in IMR32 clones with a BIRC5 cDNA construct under control of a constitutively active CMV promoter. In two independent IMR32-BIRC5 clones this resulted in rescue of the YM155-induced loss of cell viability (fig 2d). If these cells were also rescued from apoptosis induction we verified BIRC5 expression and PARP cleavage by Western blot and found a clear partial rescue from apoptosis (suppl fig 1c). This confirms that the apoptotic response after YM155 exposure results from targeted inhibition of BIRC5.

<table>
<thead>
<tr>
<th>cell lines</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR32</td>
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<tr>
<td>AMC691T(^1)</td>
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</tr>
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</tr>
<tr>
<td>CHP134</td>
<td>3</td>
</tr>
<tr>
<td>AMC700B(^1)</td>
<td>3</td>
</tr>
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<td>SMSKCNR</td>
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<tr>
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<tr>
<td>GIMEN</td>
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</tr>
<tr>
<td>LAN5</td>
<td>6</td>
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<tr>
<td>AMC 691B(^1)</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>NMB</td>
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</tr>
<tr>
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<td>SKNSH</td>
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<td>SKNFI</td>
<td>10000</td>
</tr>
<tr>
<td>TR14</td>
<td>10000</td>
</tr>
</tbody>
</table>

**ABCB1 is the most predictive gene for YM155 resistance**

These findings establish YM155 as an effective targeted compound in a series of neuroblastoma cell lines. However, in the full panel of 23 neuroblastoma cell lines the IC50 varied from 0.5 nM up to 10,000 nM and 9 out of 23 cell lines were relatively resistant to YM155 with an IC50 >200nM (table 1). We used the R2 bioinformatic platform, which contains Affymetrix mRNA expression data of 23 neuroblastoma cell lines, to identify genes that might predict or even cause resistance to YM155. Differential expression analysis between the 5 most sensitive versus the 5 most resistant classical cell lines, based on IC50 values, showed that **ABCB1** (MDR1) was the most differentially expressed gene in the analysis (p<0.02 Student T-test after FDR correction). **ABCB1** is an outlier as revealed from the volcano-plot of all genes, indicating its significance (fig 3a). Cell lines with a high **ABCB1** expression were resistant to YM155, whereas

<table>
<thead>
<tr>
<th>Table 1: Most neuroblastoma cell lines are sensitive to YM155.</th>
</tr>
</thead>
<tbody>
<tr>
<td>The IC50 values were calculated from the curves as presented in Fig 2a. The IC50 values for YM155 of all 23 neuroblastoma cell lines and 4 TIC lines are shown in the second column of this table. (^1) TIC-lines</td>
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</tbody>
</table>
Figure 1: BIRC5 shRNA induces apoptosis in neuroblastoma cell lines

(A) 72 hours after transduction with BIRC5 shRNA or SHC002 pictures were made with a 100x magnitude.

(B) Protein lysates were made of the cells of Fig 1a. Western blots were incubated with BIRC5, PARP, and Actin antibodies.
cell lines with a low ABCB1 expression were sensitive (fig 3b).

**Targeted ABCB1 silencing can restore YM155 sensitivity in resistant cell lines**

To confirm the functional importance of ABCB1, we combined targeted inhibition of the multidrug resistance pump with YM155 treatment. Cyclosporine can effectively and specifically inhibit ABCB1\(^{27,31}\). Eight YM155 resistant cell lines and one sensitive cell line as a control were treated with 5 µM of cyclosporine 1 hour prior to treatment with YM155. This resulted in a strong increase of sensitivity to YM155 of all cell lines with a high ABCB1 expression (suppl fig 2a). Crystal Violet assays revealed that cyclosporine pretreated SKNSH cells survived much less efficient than YM155 only treated cells (suppl fig 2b). MTT-assays showed that cyclosporine reduced the IC50 values of all cell lines with ABCB1 expression by 27 up to 695 fold (fig 4a, table 2). In SJNB12, which has a very low expression of ABCB1, co-incubation with cyclosporine did not result in a change of YM155 sensitivity (table 2). Western blot analysis of SKNSH, UHGNP and SHY5Y demonstrated that BIRC5 was inhibited when cells were pretreated with cyclosporine, but not when cells were treated with YM155 alone. In addition, cyclosporine pretreated cells showed sensitization to YM155 by an induction of PARP cleavage (fig 4b). Apoptosis was confirmed by FACS analysis, which showed that the apoptotic sub G1 fraction strongly increased in SKNSH (6 fold) and SHSY5Y (12 fold) when pretreated with cyclosporine before addition of YM155 (fig 4c).

We also knocked down ABCB1 in SKNSH with 2 lentiviral shRNAs targeting different parts of the coding sequence of ABCB1, which confirmed our findings with cyclosporine pretreated cells. The IC50 of SKNSH decreased from 370 nM in the untransduced control and 347 nM in the cells transduced with SHC002 to 35 and 29 nM in the cells transduced with either of the ABCB1 shRNAs (fig 4d). Knockdown

<table>
<thead>
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<th>IC50 (nM)</th>
<th>IC50 + cyclo (nM)</th>
<th>ratio</th>
<th>ABCB1 expression</th>
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<td>155</td>
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<td>747</td>
</tr>
<tr>
<td>UHGNP</td>
<td>1027</td>
<td>7</td>
<td>147</td>
<td>655</td>
</tr>
<tr>
<td>SHSY5Y</td>
<td>1282</td>
<td>8</td>
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<td>SKNSH</td>
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</tr>
<tr>
<td>SKNB</td>
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<td>SJNB12</td>
<td>78</td>
<td>66</td>
<td>1</td>
<td>12</td>
</tr>
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</table>

**Table 2: Resistant cell lines can be sensitized by Cyclosporine**

The table represents the IC50 values for YM155 of all cell lines of the panel with ABCB1 expression without and with pretreatment with cyclosporine (2nd and 3rd column), the ratio between these two values (4th column) and the ABCB1 RNA expression (5th column).
Figure 2: YM155 induces apoptosis in most neuroblastoma cell lines

(A) IC50 curves of SKNAS, IMR32 and SMSKCNR 48 hours after YM155 treatment are shown. The Y-axis represents the percentage of cell survival; the X-axis represents the concentration YM155 in nM.

(B) Western blots of SKNAS and IMR32 were incubated with BIRC5, PARP and actin antibodies. The concentrations YM155 used are shown above the blots in nM.

(C) FACS analysis of SKNAS, IMR32 and SMSKCNR 72 hours after treatment with YM155. The percentage of the sub G1 (apoptotic) fraction is presented in the graph.

(D) MTT assay of IMR32 cells that were stably transduced with BIRC5 over expression construct (IMR32-Surv-7 and IMR32-Surv-10) and treated with YM155 for 24 hours. The Y-axis represents the percentage of cell survival; the X-axis represents the concentration YM155.
of both ABCB1 shRNAs was validated as shown in fig 4e.

Clinical predictions with YM155 related biomarkers
ABCB1 is a multi drug resistance pump which is involved in chemoresistance in many types of cancer. Our results indicate that ABCB1 is a potential biomarker for efficacy. The ABCB1 mRNA expression pattern in neuroblastoma cell lines suggests dichotomy. This is to a lesser extend reflected in the neuroblastoma tumor series as shown in suppl fig 3a. We chose a cut-off value of 200 nM (as indicated in the figure) because at this value the slope of the samples ordered by ABCB1 expression was the highest. Most interestingly, the subset of tumors with low ABCB1 expression levels tends to correlate with prognostic factors such as age, stage, survival and MYCN amplification (suppl fig 3a). Also, children with a tumor with low ABCB1 expression have a poor prognosis according to the Kaplan Meier curve (p < 0.02 after Bonferroni correction) (suppl fig 3b). This suggests that patients with a poor
prognosis are likely to be sensitive to YM155. In addition, we investigated if BIRC5 could be a predictor for sensitivity. However, we did not find a correlation between the IC50 to YM155 and BIRC5 expression in our cell line panel (suppl fig 3c) and BIRC5 can therefore not be used as a predictor for sensitivity in neuroblastoma patients. This also holds true if we exclude the cell lines with high ABCB1 expression (data not shown).

Discussion

We conclude that 14 out of 23 neuroblastoma cell lines are sensitive to YM155 in the low nM range. Most small molecule compounds used in anticancer treatment are known to inhibit a variety of genes. The lack of specificity is an important cause of the severe side effects of these compounds and it is well established that blocking a single target with high potency minimizes the side effects.\textsuperscript{32} YM155 was picked up by a screen that was designed to select compounds in a chemical compound library efficiently inhibiting the BIRC5 promoter.\textsuperscript{21} This resulted in a highly effective BIRC5 suppressant as was validated in our experiments. We were able to rescue YM155 induced apoptosis by BIRC5 over-expression, which suggests that YM155 is a highly specific BIRC5 suppressant. These findings establish YM155 as an interesting compound for treatment of neuroblastoma patients.

Most interestingly, also the 4 TIC lines we tested were shown to be very sensitive to YM155. These TIC lines are cultured in neural stem cell medium, grow in spheroids and have been cultured only for a limited number of passages. Therefore these cells are thought to be a better representation of in vivo neuroblastoma tumors.\textsuperscript{33} In addition these cells have been shown to have increased tumorigenicity in in vivo models.

Analysis of the differential expression between the 5 most sensitive and the 5 most resistant classical cell lines revealed that increased expression of ABCB1 is a good predictor for insensitivity to YM155. The other ABC transporters that are known as a multi drug resistant pump (ABCC1 and ABCG2) did not reveal any significant correlation between these two groups (data not shown). Inhibition of ABCB1 with cyclosporine resulted in sensitization of all resistant cell lines with ABCB1 expression, which was confirmed by shRNA mediated silencing of ABCB1. Cyclosporine is originally used as an immunosuppressant drug in patients after
Figure 4: Resistant cell lines can be sensitized by Cyclosporine or ABCB1 shRNA

(A) MTT-assay was performed 72 hours after treatment with YM155. 3 cell lines that were treated with YM155 with or without cyclosporine are shown. The Y-axis represents the percentage cell survival; the X-axis represents the concentration YM155 in nM. The dotted line is the curve for YM155 without cyclosporine; the continuous line is the curve for the combination of YM155 and cyclosporine. (B) Western blots were incubated with BIRC5, PARP and actin antibodies. 3 Cell lines are shown, the concentration YM155 is depicted in nM. (C) FACS analysis of SKNSH and SHSY5Y treated with YM155 with or without cyclosporin. The percentage of the sub G1 (apoptotic) fraction is presented in the graph. (D) SKNSH cells were transduced with 2 different ABCB1 shRNAs (B5 and B7) or with SHC002 (control virus) 72 hours after transduction cells were treated with a concentration series of YM155. 72 hours after treatment an MTT-assay was performed as described previously. The Y-axis represents the percentage of cell survival; the X-axis represents the concentration YM155 in µM. (E) Knockdown of ABCB1 protein in SKNSH 72 hours after transduction with both ABCB1 shRNAs was checked by Western blot. Blots were incubated with ABCB1 and Actin antibodies.
organ transplantation. It is also an active inhibitor of ABCB1; however for this use high concentrations were needed and found to be toxic in combination treatment presumably because cyclosporine induced sensitization of the bone marrow to chemo therapy.\textsuperscript{27,34} Currently, new inhibitors of ABC transporters are in clinical development, such as PSC833, V-104, tarquidar and ONT-093.\textsuperscript{26-28} After clinical implementation these compounds could be combined with YM155.

Still, targeted ABCB1 inhibition is currently not possible in a clinical setting. The over-expression of ABCB1 in neuroblastoma however can be used as a selection biomarker. \textit{BIRC5} is over-expressed in almost all high risk neuroblastoma and in principle serves as drug target in these patients. As high \textit{ABCB1} expression prevents effective targeting, we propose to select ABCB1 negative patients for clinical testing of YM155. Most interestingly, this group of patients tends to have a very poor prognosis and new therapeutic options are urgently needed in this specific subgroup.

Before YM155 can be used in a Phase I/II clinical trial in neuroblastoma patients, the compound needs to be validated in a neuroblastoma mouse model. New compounds will only be used in neuroblastoma patients in combination with the currently used cytostatics. Therefore these interactions need to be evaluated. The knowledge that mitotic catastrophe is involved in the apoptotic response after \textit{BIRC5} knockdown can also guide compound combination strategies.\textsuperscript{11} Simultaneous inhibition of other genes in the same signal transduction pathway could lead to additional or synergistic effects. For example AURKB inhibitors could potentially enhance the effect of \textit{BIRC5} inhibition as they both are part of the chromosomal passenger complex. Mitotic catastrophe results in an apoptotic response via mitochondrial release of pro-apoptotic proteins. Sensitization of this downstream signal transduction pathway by BCL2 inhibitors or SMAC mimetics might lead to synergism with a \textit{BIRC5} inhibitor. Based on in vivo experiments and on knowledge about the efficacy of YM155 combined with other drugs, a Phase I/II clinical trial can be designed.

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Reference List

Supplementary Figures

Supplementary figure 1:
(A) SKNAS and IMR32 cells were treated with a concentration series of YM155 24 hours after plating. Pictures were made 48 hours after treatment with a 100x magnitude. (B) 48 hours after treatment with YM155, SKNAS and SMSKCNR cells were fixed with methanol and stained with crystal violet (concentrations depicted in each well). (C) Left: Western blot of IMR32 and IMR32 with BIRC5 over-expression treated with YM155. Concentrations YM155 are depicted below in nM. Blots were stained with BIRC5, PARP and Actin antibodies. These lanes were on the same blot. BIRC5 expression was quantified and corrected for actin which is represented in the middle panel. The Y-axis represents the percentage BIRC5 expression relative to IMR32 without YM155. On the X-axis the concentration YM155 is shown in nM. The dark bars represent IMR32 and the light bars represent IMR32-Surv-10. Right: PARP cleavage was quantified and corrected for actin. On the Y-axis the percentage of cleaved PARP in IMR32-Surv-10 relative to IMR32 treated with the same concentration YM155 is shown. The X-axis represents the concentration YM155 in nM.
Supplementary figure 2:
(A) Cells were treated with cyclosporine 24 hours after plating. After 1 hour of incubation YM155 was added. Pictures were made 48 hours after treatment with a 100x magnitude. (B) SKNSH cells were treated with cyclosporine prior to treatment with YM155 (concentrations depicted above the wells) and fixed with methanol and stained with crystal violet.
Supplementary figure 3:

(A) The left panel indicates the Relative ABCB1 RNA expression based on MAS5.0 corrected Affymetrix Micro-array data (Y-axis) of 88 neuroblastoma tumors (X-axis) ordered by ABCB1 expression. ABCB1 expression was determined by two different probe sets. Every dot represents one sample; below every dot clinical information is given. Age: red is >= 1 year; green is < 1 year. Alive: red = deceased, green = alive. Stage: red = stage 3 or 4, green = stage 1 or 2, blue = stage 4S. MYCN: red = amplified, green = not amplified. The right panel shows the ABCB1 RNA expression levels in the neuroblastoma cell lines of our panel ordered by ABCB1. Both Y-axes represent different probe sets for ABCB1; cell lines are depicted below together with the NMYC status: red = amplified, green = not amplified, grey = not determined. The black line indicates the proposed cut-off for YM155 sensitivity prediction. (B) Kaplan Meier curve of 88 neuroblastoma tumors based on Affymetrix Micro-array RNA expression data. On the Y-axis the overall survival probability is presented and on the X-axis the time in months after diagnosis. The blue line represents the patients with a tumor with high ABCB1 expression; the red line represents the patients with a tumor with low ABCB1 expression. The P-value is under 0.02 after Bonferroni correction with an RNA expression cutoff of 39.1. The cutoff was chosen that gives the lowest P-value with at least 10 samples in one group. (C) The red bars represent IC50 levels for all neuroblastoma cell lines tested; the blue dots represent BIRC5 RNA expression levels as determined by Affymetrix microarray. The cell lines are depicted below.