EUROPEAN JOURNAL OF CANCER 48 (2012) 763–771

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

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ARTICLE INFO

Article history:
Available online 14 November 2011

Keywords:
Neuroblastoma
BIRC5
Apoptosis
YM155
ABCB1
Cancer

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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doi:10.1016/j.ejca.2011.10.012

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.

3. Results

3.1. *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 h 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

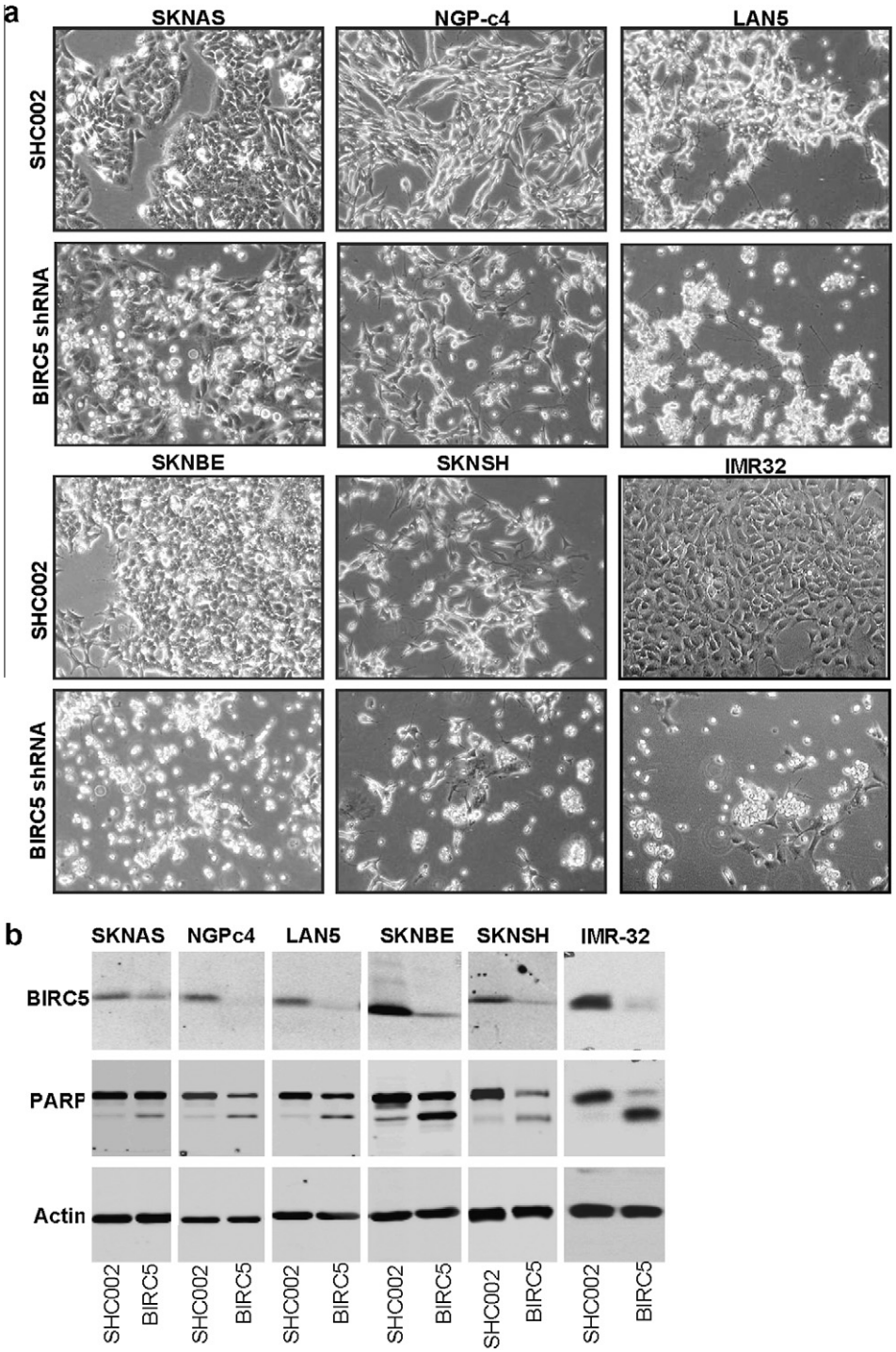

Fig. 1 – BIRC5 shRNA induces apoptosis in neuroblastoma cell lines. (a) Seventy-two hours after transduction with BIRC5 shRNA or SHC002 pictures were made with a 100× magnitude. (b) Protein lysates were made of the cells of (a). Western blots were incubated with BIRC5, PARP, and Actin antibodies.

as a potential therapeutic intervention in neuroblastoma tumour cells and we therefore decided to test the efficacy of the small molecule BIRC5 suppressant YM155 in neuroblastoma cell lines.

3.2. 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 four newly isolated TIC-lines to YM155. These cells were isolated directly from patient tumour 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).

Table 1 – Most neuroblastoma cell lines are sensitive to YM155. 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 four TIC lines are shown in the second column of this table.

Cell lines	IC50 (nM)
IMR32	0.5
AMC691T[a]	1.5
SKNAS	2
CHP134	3
AMC700B[a]	3
SMSKCNR	4
AMC700T[a]	5
GIMEN	6
LAN5	6
AMC 691B[a]	7
LAN1	10
SHEP21N	12
AMC106	14
NMB	23
N206	35
SHEP2	39
SJNB8	73
SJNB12	78
SJNB6	192
SJNB10	306
SKNBE	356
NGP	375
SJNB1	496
UHGNP	1027
SHSY5Y	1282
SKNSH	6188
SKNFI	10,000
TR14	10,000

[a] TIC-lines.

3.3. 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 h 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.

3.4. 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 nine out of 23 cell lines were relatively resistant to YM155 with an IC50 >200 nM (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 five most sensitive versus the five 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 cell lines with a low ABCB1 expression were sensitive (Fig. 3b).

3.5. Targeted ABCB1 silencing can restore YM155 sensitivity in resistant cell lines

To confirm the functional importance of ABCB1, we combined targeted inhibition of the multi-drug 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 h 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 efficiently than YM155 only treated cells

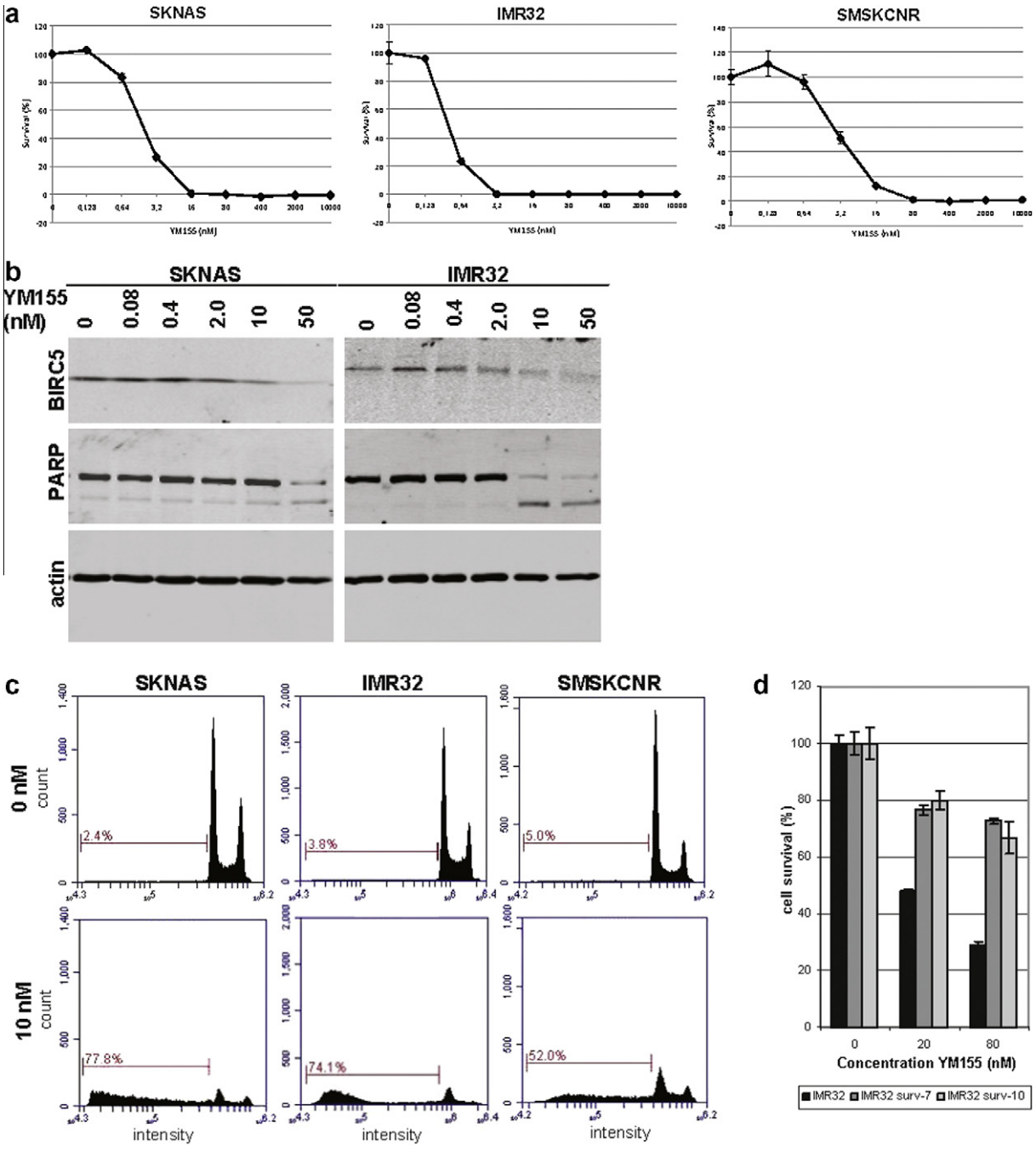

Fig. 2 – YM155 induces apoptosis in most neuroblastoma cell lines. (a) IC50 curves of SKNAS, IMR32 and SMSKCNR 48 h after YM155 treatment are shown. The Y-axis represents the percentage of cell survival; the X-axis represents the concentration YM155 in nM. (b) Western blots of SKNAS and IMR32 were incubated 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 h 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 h. The Y-axis represents the percentage of cell survival; the X-axis represents the concentration YM155.

(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 and 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 sensitisation 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

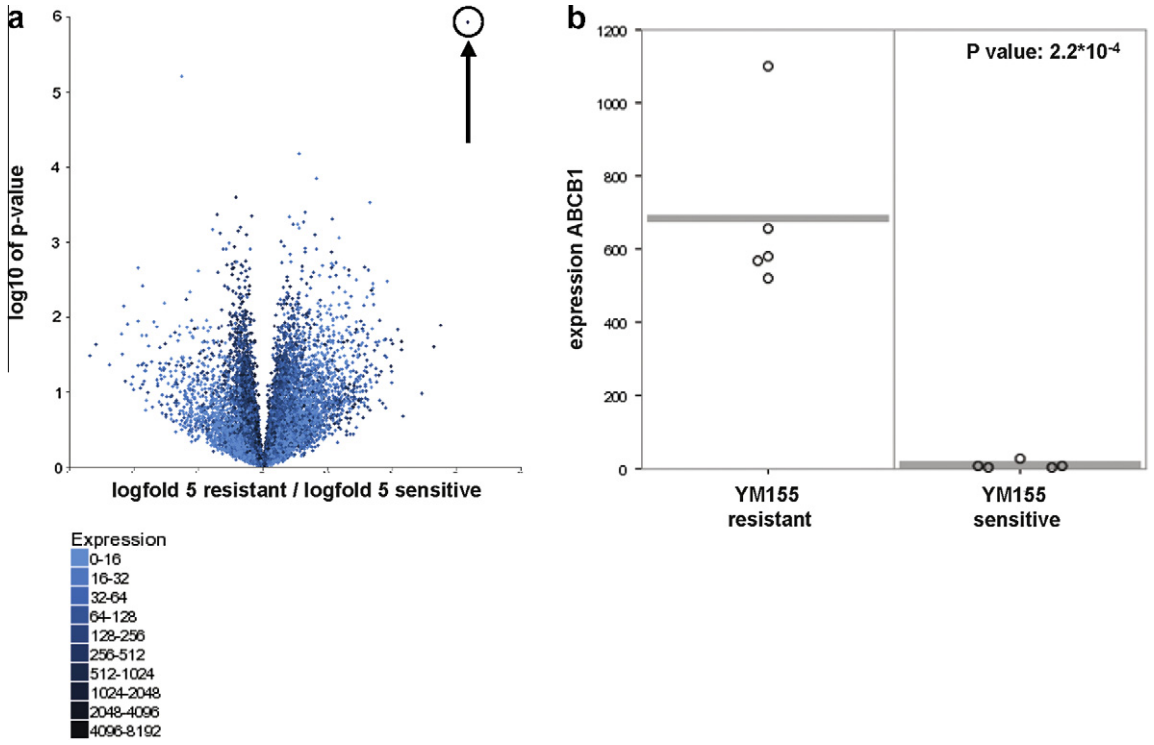

Fig. 3 – ABCB1 is the most differentially expressed gene between YM155 sensitive and resistant cell lines. (a) Volcano-plot of all genes based on Affymetrix Micro-array RNA expression data. The Y-axis represents the log10 of the *P*-value. The X-axis represents the log-fold of the five most resistant cell lines (TR14, SKNFI, SKNSH, SHSY5Y, UHGNP) divided by the log-fold of the five most sensitive cell lines (IMR32, SKNAS, CHP134, SMSKCNR, GIMEN). The arrow indicates ABCB1, which is the only significantly differentially expressed outlier. (b) Relative ABCB1 RNA expression based on MAS5.0 corrected Affymetrix Micro-array data (Y-axis) of the five most sensitive versus the five most resistant cell lines (X-axis). The horizontal line represents the average expression.

increased in SKNSH (6-fold) and SHSY5Y (12-fold) when pre-treated with cyclosporine before addition of YM155 (Fig. 4c).

We also knocked down ABCB1 in SKNSH with two lentiviral shRNAs targeting different parts of the coding sequence of ABCB1, which confirmed our findings with cyclosporine pre-treated 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 of both ABCB1 shRNAs was validated as shown in Fig. 4e.

3.6. *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 extent reflected in the neuroblastoma tumour 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 tumours 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 tumour 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).

4. 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 minimises the side-effects.[32] YM155 was picked up by a screen that was designed to select compounds in a chemical compound library efficiently inhibiting the BIRC5 promoter.[21] This resulted in a highly effective BIRC5 suppressant as was validated in our experiments. We were able to rescue YM155

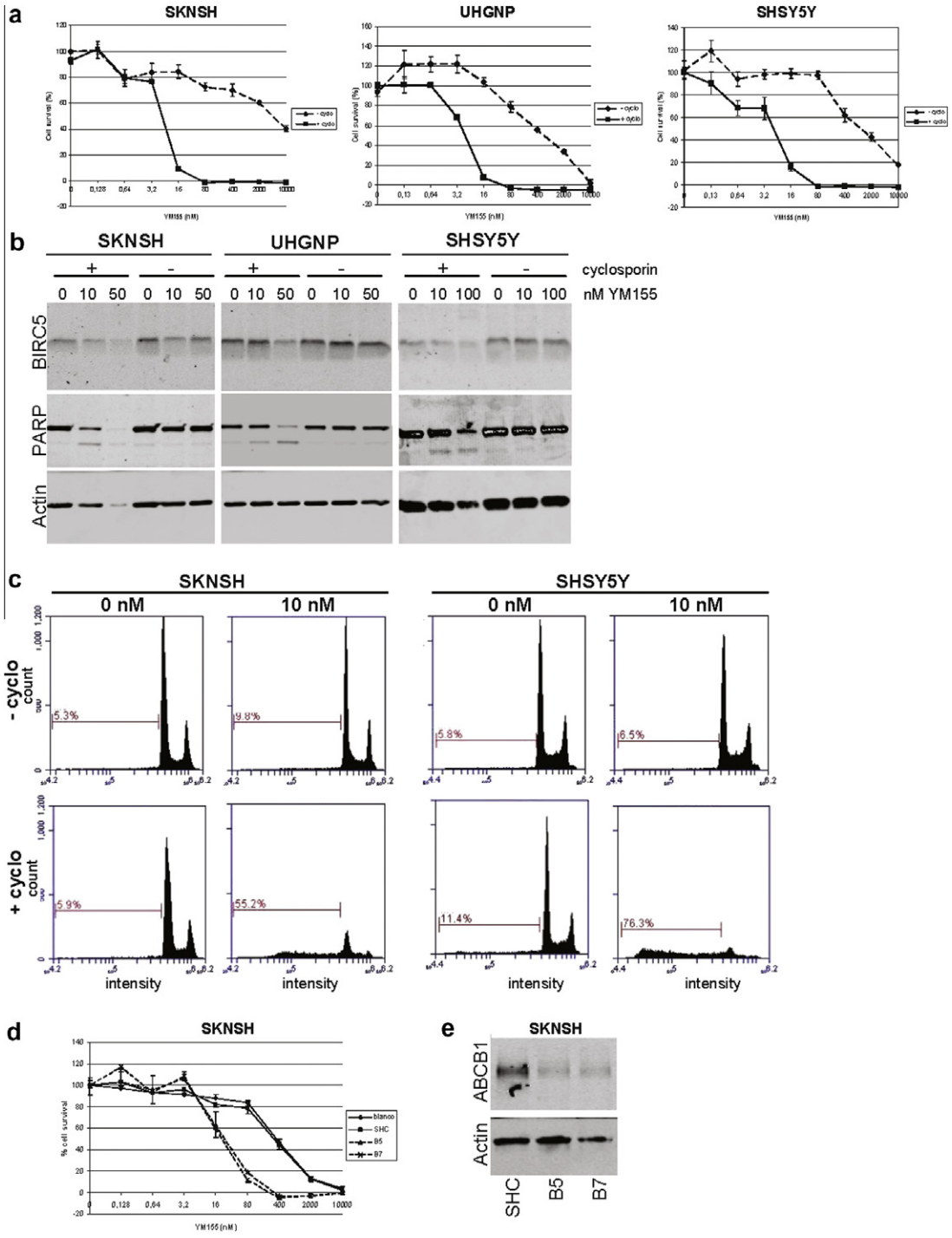

Fig. 4 – Resistant cell lines can be sensitised by cyclosporine or ABCB1 shRNA. (a) MTT-assay was performed 72 h after treatment with YM155. Three cell lines that were treated with YM155 with or without cyclosporine are shown. The Y-axis represents the percentage cell survival; the X-axis represents 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. Three 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 two different ABCB1 shRNAs (B5 and B7) or with SHC002 (control virus). Seventy-two hours after transduction cells were treated with a concentration series of YM155. Seventy-two 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 h after transduction with both ABCB1 shRNAs was checked by Western blot. Blots were incubated with ABCB1 and Actin antibodies.

Table 2 – Resistant cell lines can be sensitised 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).

cell lines	IC50 (nM)	IC50 + cyclo (nM)	ratio	ABCB1 expression
SJNB10	306	9	36	155
SKNBE	356	7	51	604
SJNB1	496	2	248	747
UHGNP	1027	7	147	655
SHSY5Y	1282	8	169	522
SKNSH	6188	9	695	568
SKNFI	>10,000	366	27	581
TR14	>10,000	40	249	1100
SJNB12	78	66	1	12

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 four 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 tumours.[33] In addition these cells have been shown to have increased tumourigenicity in *in vivo* models.

Analysis of the differential expression between the five most sensitive and the five 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 sensitisation 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 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 sensitisation of the bone marrow to chemo therapy.[27,34] Currently, new inhibitors of ABC transporters are in clinical development, such as PSC833, V-104, tarquidar and ONT-093.[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. BIRC5 is over expressed in almost all high risk neuroblastoma and in principle serves as drug target in these patients. As high 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 BIRC5 knockdown can also guide compound combination strategies.[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 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. Sensitisation of this downstream signal transduction pathway by BCL2 inhibitors or SMAC mimetics might lead to synergism with a 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.

Conflict of interest statement

None declared.

Acknowledgement

This research was supported by grants from KIKA foundation, SKK and Netherlands Cancer Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.10.012.

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