Preclinical Evaluation of Combined Targeted Approaches in Malignant Rhabdoid Tumors

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Abstract. *Background/Aim: Rhabdoid tumors (RT) are aggressive pediatric tumors, which show poor prognosis despite use of multimodal intensive therapy. In these tumors, several different oncogenic pathways and epigenetic regulators (like CDK4/6-cyclinD-Rb- signaling, EZH2, histone deacetylases) are contemporaneously deregulated as a consequence of biallelic SMARCB1/SNF5/INI1 alterations. Since these tumors are highly resistant to current therapies, alternative treatment strategies are urgently required. Materials and Methods: In this study, we evaluated cytotoxic effects (by MTT tests) of small molecular compounds, which specifically target these deregulated pathways, using either single-drug or combined approaches. Half-maximal inhibitory concentration (IC_{50}) and combined index (CI) were calculated. Results: All target-directed inhibitors blocked cell growth of three different rhabdoid tumor cell lines in vitro. Several combinations of those target-specific drugs synergistically inhibited cell proliferation of rhabdoid tumors. Conclusion: Supporting earlier reports, combined target-directed approaches are a promising tool for the therapy of malignant rhabdoid tumors.*

Rhabdoid tumors (RT) are aggressive cancers affecting predominantly infants and young children. These neoplasms occur in different anatomical localizations, mainly in the kidneys (RTK, rhabdoid tumors of the kidney), in the brain (AT/RT, atypical teratoid, rhabdoid tumors) and in the soft tissues (MRT, malignant rhabdoid tumors) (1, 2).

The prognosis of patients has improved significantly in the last years as a result of implementing treatment protocols specifically designed for these tumor entities (3). Nevertheless, overall survival (OS) of patients, especially in relapses of rhabdoid tumors, is still poor, despite using incisive multimodal chemotherapeutic, radiotherapeutic and surgical interventions. Further intensification of therapy does not seem to improve prognosis but increases the rate of toxic side-effects (4) making the development of alternative therapeutic approaches necessary.

The majority of RTs exhibit homozygous deletions or mutations of the *SMARCB1* (also known as *hSNF, INI1, BAF47*) tumor suppressor gene, localized on chromosome 22q11.2 (5). This gene encodes one of the core subunits of the human ATP-dependent chromatin remodeling complex SWI/SNF, which is involved in modulation of accessibility of chromatin to transcription factors and, thus, in regulation of gene transcription, as well as in a wide variety of other cellular processes (6).

Biallelic loss of *SMARCB1* leads to deregulation of cell signaling pathways implicated in oncogenesis like CDK4/CDK6/cyclinD1 (7), aurora kinase A (8) or the Sonic hedgehog pathway (SHH) (9). Deregulated epigenetic mechanisms (*e.g.* EZH2 (10) and HDACs (11)) have also been described in these tumor entities. In addition, loss of *SMARCB1* in RT led to increased phosphorylation of eIF2α, a central cytoplasmic unfolded protein response (UPR) component, suggesting a role for the UPR in these tumors (12).

In a previous study, we could demonstrate that targeting one deregulated pathway in RT might lead to further pronounced up-regulation of a second mechanism of tumorigenesis (13). This observation makes combined therapeutical targeted approaches reasonable. The purpose of our study was to investigate the cytotoxic activity of specific molecular inhibitors affecting known deregulated pathways in RT. Therefore, cytotoxicity on different tumor cell lines derived from primary RT was explored by using single and combined approaches of multiple target-specific small molecular inhibitors. Since RT presents contemporaneously a deregulation of not only one, but multiple molecular pathways, we hypothesized that synergistic effects of these substances may be a valuable tool for their treatment.

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Materials and Methods

Cell lines. RT cell lines LM (RT of liver), G401 (RT of the kidney (RTK) and A204 (RT of the liver) were cultured in DMEM high-glucose formulation (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (South American, Invitrogen, Carlsbad, CA, USA), 2% glutamine (Invitrogen) and 1% of penicillin/streptomycin (Invitrogen). The cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2. A204 and G401 were obtained from ATCC (Wesel, Germany). LM cells were a gift from Prof. Handgretinger (Tübingen, Germany). The identity of all cell lines was verified using single-tube polymerase chain reaction (ST-PCR). All experiments in this publication were performed using at least three independent replicates.

Inhibitors. These inhibitors were used in this study: suberoylanilindehydroxamic acid (SAHA), histondeacetylase (HDAC) inhibitor (SML-0061; Sigma, Taufkirchen Germany,); DZNep, EZH2 inhibitor (#252790; Merck Millipore, Darmstadt, Germany); bortezomib (BZ), proteasome inhibitor (#5.04314.0001; Merck Millipore), CDK4 inhibitor (#219476; Merck Millipore); MLN 8054, aurora kinase inhibitor (Seleckchem, Huston, TX, USA).

All inhibitors were reconstituted according to manufacturers' recommendations either in ethanol or dimethyl sulfoxide (DMSO). All compounds were stored until further usage as a 10 mM solution.

Cytotoxicity assay. Cell suspensions (5,000 cells/100 μl) were seeded into four 96-well-plates. Cells were allowed to reach exponential growth before 100 μl of cell culture medium containing the drugs at different concentrations were added. Each drug concentration (0, 0.01, 0.1, 1, 10 and 100 μM) was tested in 3 biological replicates. For experiments with combined treatment (Table II), we used compound 1 in increasing concentrations as in single compound experiments (0, 0.01, 0.1, 1, 10 and 100 μM). DZNep was assayed in a range from 0.001 to 10 μM. Compound 2 was used at 1/10 of the concentration of compound 1. After 72 h, cells were incubated for 4 h with 10 μl MTT reagent (5 mg/ml MTT dissolved in PBS). Metabolically active cells cleaved the yellow tetrazolium salt to a purple formazan dye. The resulting crystals were dissolved in 100 μl isopropanol - 0.04 N HCl. The specimen was evaluated spectrophotometrically at 570 nm and a reference of 650 nm using a Multiskan Ascent multiplate reader (Labsystems, Helsinki, Finland).

Analysis of combined drug effects on cytotoxicity. To evaluate drug combination effects we analyzed cytotoxicity assay data using the median effect method by Chou and Talalay (14). The fraction of unaffected cells was defined as the proportion of living cells compared to the control. The combination index (CI) indicates synergism if CI <1, antagonism for CI >1 and an additive effect for CI=1. Values of the CI were determined at the half-maximal inhibitory concentration (IC_{50}) concentration (fraction affected=0.5). The method was implemented in the statistical software R (Version 2.15.1; https://cran.r-project.org/bin/windows/base/old/2.15.1/).

Results

Small molecular compounds, which target deregulated signaling, inhibit proliferation of rhabdoid tumor cells. In RT multiple signaling, pathways, as well as epigenetic modulating mechanisms, have been found to be deregulated (7, 10, 11, 15). The aim of this study was to evaluate the cytotoxic effects of different small molecular compounds that specifically inhibit these altered pathways. The CDK4/6-cyclinD-Rb pathway, targeted by a specific CDK4 inhibitor (CDK4i) and aurora kinase A inhibited by MLN 8054 are both implicated in cell cycle progression and, thus, in cellular proliferation. Epigenetic regulators were targeted by SAHA inhibiting HDACs and by DZNep inhibiting the histone methyltransferase EZH2, which silences genes by trimethylating histone H3K27. BZ was included in this study as a proteasome inhibitor because loss of *SMARCB1* led to increased phosphorylation of eIF2α in rhabdoid tumors (12).

Table I. *IC_{50} values of single drug treatments. Summarizes results of proliferation assays (after 72 h of treatment) of five different small molecular inhibitors (bortezomib, CDK4i, MLN8054, SAHA, DZNep) in three different rhabdoid tumor cells lines (A204, G401, LM).*

	Cell line		
IC_{50} (μM)	A204	G401	LM
Bortezomib	0.02	0.07	0.01
CDK4i	3.07	4.89	1.01
MLN	57.44	6.47	6.83
SAHA	16.4	7.27	3.28
DZNep	3.54	3.45	0.26

IC_{50}, Growth inhibition by 50%.

In proliferation assays, all used small molecular compounds were able to inhibit proliferation of all three different RT cell lines (A204, G401 and LM) in a nanomolar to micromolar range (Figure 1 and Table I). All cell lines tested exhibited a significant dose-dependent reduction of cell numbers after the treatment. G401 and LM were more sensitive to all the inhibitors evaluated, in comparison to the RT cell line A204.

Administration of BZ very strongly decreased cell proliferation of all three cell lines being the most cytotoxic compound of all tested substances with an IC_{50} ranging from 0.01 to 0.07 μM, depending on the studied cell line.

Treatment with DZNep was able to inhibit very efficiently cell proliferation, especially of LM cells, which presented an IC_{50}=0.26 μM.

Combinations of small molecular inhibitors, which target deregulated signaling pathways in rhabdoid tumor, act synergistically on blocking tumor cell proliferation. In this study, we aimed to evaluate *in vitro* combined approaches of compounds targeting different deregulated signaling pathways in RTs. We included five different compounds (BZ, CDK4i, DZNep, MLN8054 and SAHA) in ten different

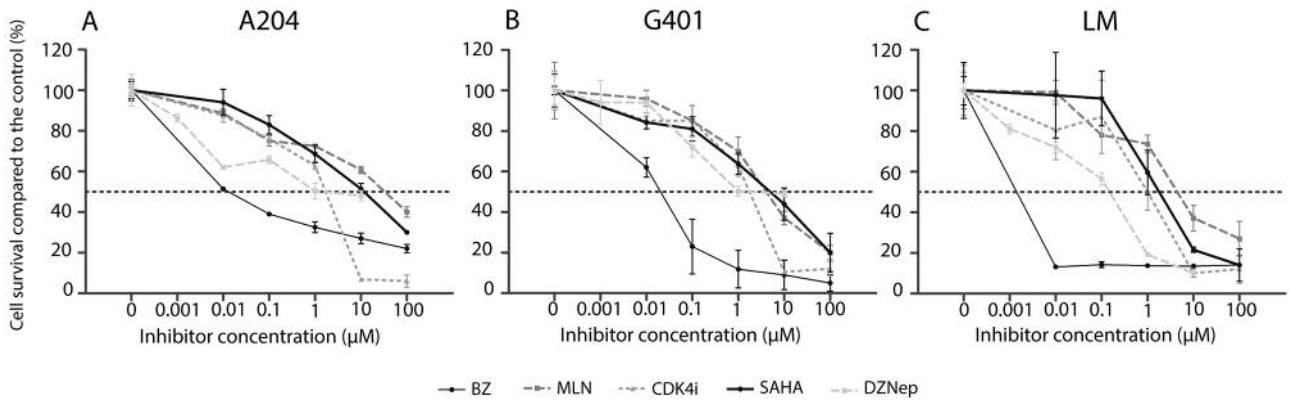

Figure 1. *Effect of single-compound treatment on proliferation assays of RT cell lines. Concentration-dependent response to treatment with the indicated inhibitors performed on rhabdoid tumor cell lines A204 (A), G401 (B) and LM (C) after 72 h. Dotted line indicates 50% of living population. Every experiment was performed in triplicate.*

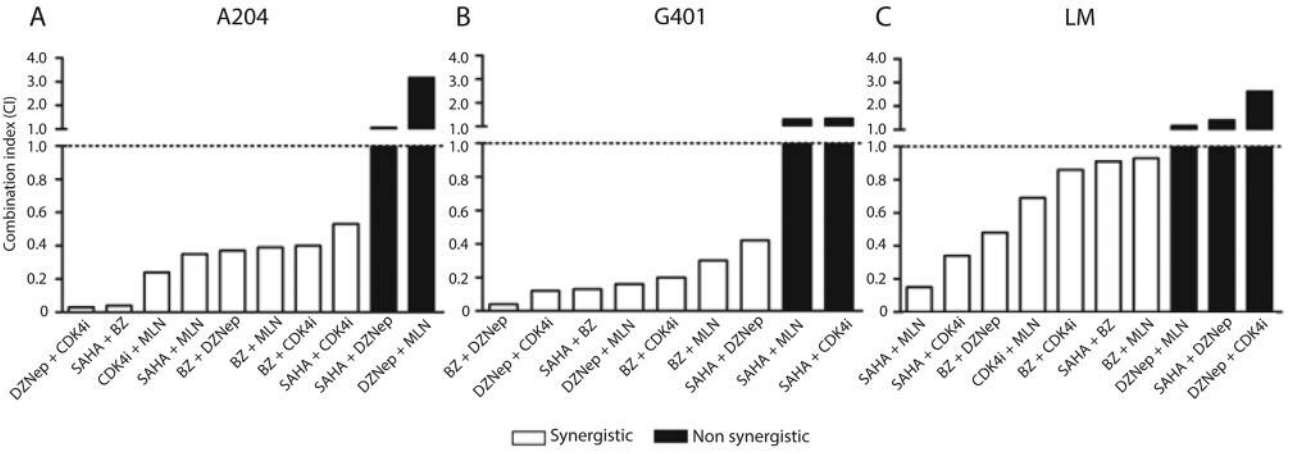

Figure 2. *Simultaneous treatment with different target molecule inhibitors act synergistically on inhibiting cell growth of RT. Combined index (CI) of three rhabdoid tumor cell lines A204 (A), G401 (B) and LM (C). Proliferation assay (after 72 h of treatment) of stated combination were used. CI below 1.0 indicated synergistic effects; CI equal or above 1.0 indicated non-synergistic (additive or antagonistic) effects.*

combinations and tested them on three different RT cell lines (G401, A204, LM). Five of those drug combination inhibited synergistically tumor growth in all cell lines used in these experiments (BZ+CDK4i; BZ+DZNep; BZ+MLN; CDK4i+MLN; SAHA+BZ) (Figure 2 and Table II). Interestingly, in the other five drug combinations, we observed differences between the three treated cell lines. A204 and LM cells (both liver-derived) showed comparable synergistic/antagonistic drug profiles of the different compound combinations (*e.g.* antagonistic: DZNep+MLN; SAHA+DZNep). On G401, all tested combinations showed synergistic effects in tumor cell growth inhibition except of SAHA+DZNep and SAHA+MLN, which acted antagonistically.

Discussion

RT are aggressive pediatric malignancies characterized by the biallelic inactivation of the tumor suppressor *SMARCB1* (16), one of the core subunits of the SWI/SNF complex. Alterations in the SWI/SNF complex have been found to be implicated in cell differentiation, developmental diseases and cancer (17).

Different mechanisms driving oncogenesis, including cell signaling transduction and cell cycle regulating pathways, like CyclinD1-Rb (7), aurora kinase A (12) and Sonic hedgehog signaling (9), as well as enzymes controlling other cellular events like proteosomal pathways, are known to be deregulated in RT. Different epigenetic mechanisms, including the trimethylation of histone H3K27 and histone deacetylation,

Table II. *IC_{50} and CI values of combinatorial drug treatments. Results summary of proliferation assays (after 72 h of treatment) of ten combinations of five different small molecular inhibitors (bortezomib, CDK4i, MLN8054, SAHA, DZNep) in three different rhabdoid tumor cells lines (A204, G401, LM). The CI values have been determined at the perspective at the respective IC_{50} value. CI<1 indicates synergism; CI>1 indicates additive or antagonistic effects. R^2 denotes the coefficient of determination of the linear regression in the median effect plot.*

	A204			G401			LM		
Treatment	IC_{50} (μM)	CI	R^2	IC_{50} (μM)	CI	R^2	IC_{50} (μM)	CI	R^2
BZ + CDK4i	0.01	0.40	0.96	0.009	0.20	0.85	0.0062	0.86	0.89
BZ + DZNep	0.01	0.37	0.92	0.002	0.04	0.92	0.0048	0.48	0.94
BZ + MLN	0.01	0.39	0.92	0.02	0.30	0.91	0.0058	0.93	0.79
CDK4i + MLN	0.79	0.24	0.97	n.d.	n.d.	n.d.	0.75	0.69	0.98
DZNep + MLN	0.81	3.17	0.84	0.76	0.16	0.94	0.55	1.17	0.93
DZNep + CDK4i	0.09	0.03	0.93	0.49	0.12	0.91	0.81	2.62	0.84
SAHA + BZ	0.01	0.04	0.88	0.09	0.13	0.96	0.06	0.91	0.89
SAHA + CDK4i	5.56	0.53	0.90	7.76	1.33	0.95	1.08	0.34	0.91
SAHA + DZNep	16.04	1.06	0.89	2.96	0.42	0.82	3.69	1.41	0.86
SAHA + MLN	6.12	0.35	0.94	8.33	1.30	0.90	0.50	0.15	0.93

IC_{50}, Growth inhibition by 50%; CI, combined index.

are altered in RTs. Consequently, substances targeting molecules implicated on these pathways have to be systematically evaluated as potential therapeutic approaches for these tumors. In this study, we performed a preclinical *in vitro* screen using multiple small molecular inhibitors for combined approaches to inhibit RT cell proliferation. In this screen, all RT cell lines showed tumor cell growth inhibition to these target directed inhibitors.

Nowadays, performing whole-genome sequencing, genome-wide gene expression and methylome analyses allow the examination of deregulated pathways for individual patients and, thus, the application of a personalized molecular targeted therapy, which may be a promising tool in the treatment of rhabdoid tumors.

In other tumor entities, including a subset of medulloblastomas (MB), individual pathways, such as the Sonic hedgehog pathway, which drive tumorigenesis (18), are detected in clinical trials for personalized treatments. On the one hand, SHH-activated MB with *PTCH* mutations has been shown to be highly responsive to SMOOTHENED (SMO) receptor antagonists (19). On the other hand, mutations in the SMO receptor during treatment, making these tumors resistant to this kind of therapy, have been reported (20-22). Due to these known mechanisms of developing resistances to target directed drugs, a combined approach makes sense in the therapy of RT. The diversity of deregulated signal pathways presented by this tumor entity, including different subgroups (23), is another argument to use combinations of target-specific inhibitors.

In summary, in this study we showed that diverse combinations of target-specific drugs exert strong synergistic effects on tumor cell proliferation inhibition of RT.

Compounds of all tested classes of inhibitors are used in clinical trials (24-27) making a rapid transfer after further preclinical evaluation into clinical trials for the treatment of RT patients feasible.

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