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Original Paper

Serum and Glucocorticoid Inducible Kinase 1-Sensitive Survival, Proliferation and Migration of Rhabdomyosarcoma Cells

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Key Words
SGK1 • Rhabdomyosarcoma • Migration • Cell proliferation • Clonal cell growth

Abstract
Background/Aims: Rhabdomyosarcoma, the most common pediatric soft tissue sarcoma, may show an intrinsic refractoriness to standard chemotherapy in advanced tumor stages, which is associated with poor prognosis. Cellular mechanisms conferring tumor cell survival and therapy resistance in many tumor types include the serum & glucocorticoid inducible kinase (SGK) 1 pathway, a kinase expressed ubiquitously with particularly strong expression in skeletal muscle and some tumor types. The present study explored whether SGK1 is expressed in rhabdomyosarcoma and, if so, whether this kinase impacts on tumor cell survival, proliferation and migration. Multiple *in vitro* techniques were used to study the role of SGK1 in rhabdomyosarcoma. ***Methods:*** The Gene Chip® Human Genome U133 Plus 2.0 Array were employed to examine SGK1 transcriptional activity in healthy muscle and rhabdomyosarcoma tissue. SGK1 transcript levels were quantified in rhabdomyosarcoma cell lines RD (embryonal subtype) and RH30 (alveolar subtype) by RT-PCR, cell viability was measured using MTT assays. Clonal cell growth was assessed via colony forming assays and migration experiments were performed in a transwell system. ***Results:*** SGK1 is expressed in embryonal and alveolar rhabdomyosarcoma tissue samples and in RD and RH30 rhabdomyosarcoma cell lines. Administration of EMD638683 – an inhibitor specific for SGK1 - decreased viability of RD and RH30 cells, enhanced the effects of the cytotoxic drug doxorubicin leading to reduced migration and decreased cell proliferation. ***Conclusions:*** SGK1 is expressed in rhabdomyosarcoma cells where it contributes to survival, therapy resistance, cell proliferation and migration. Thus, SGK1 inhibitors may be considered a therapeutic option for the treatment of therapy-resistant rhabdomyosarcoma.

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Introduction

Rhabdomyosarcoma is the most prevalent soft tissue sarcoma in children and adolescents, accounting for 5% of all pediatric tumors [1]. The two main histological subtypes of RMS are embryonal (RME) and alveolar (RMA) RMS. RMA is driven typically by the fusion proteins PAX3-FKHR or PAX7-FKHR [2]. Seventy percent of all RMS in children are diagnosed less than 10 years of age, which are mostly RME. In contrast, RMA occur mostly in adolescent and young adults [3]. Prognosis is related to the localization of the primary tumor, its histological subtype, tumor size at diagnosis, the stage of disease and the age of the patient at time point of diagnosis [4, 5]. Patients with advanced stage disease, metastatic disease and local tumor recurrence have a poor prognosis. Survival rates have not improved over the past 20 years [6, 7]. Therefore novel therapeutic strategies are urgently needed for these patients.

Mechanisms conferring tumor cell survival and therapy resistance include the serum & glucocorticoid inducible kinase SGK1, initially identified in mammary tumor cells [8-14], yet then found as being ubiquitously expressed with strong emphasis in skeletal muscle [15] and highly upregulated in different tumors such as colonic cancer [16], hepatocellular carcinoma [17, 18], breast cancer [18-20], prostate cancer [21, 22], glioblastoma [23-25], non-small cell lung cancer [26], meningiomas [27], and medulloblastoma [28]. Several studies demonstrate a pivotal role for SGK1 in cell survival, cell proliferation and migration of tumor cells [16, 29].

In addition, SGK1 was shown to be essential for IL2-dependent protection of doxorubicin induced apoptosis in kidney cancer cells, suggesting a SGK1-mediated chemotherapy resistance of tumor cells [30]. Accordingly, SGK1 knock-out mice were more sensitive to kidney failure under doxorubicin treatment [12, 31] while inhibition of SGK1 via RNA silencing increased the toxicity of chemotherapeutic drugs in cancer cells [12, 31]. The highly selective SGK1 inhibitor EMD638683 proved to be effective *in vitro* and was the first SGK1 inhibitor also effective *in vivo* [32].

The present study explored whether EMD638683 alone or in combination with the cytotoxic drug doxorubicin could decrease cell viability, proliferation and migration of rhabdomyosarcoma cells *in vitro*.

Material and Methods

Cell lines and culture conditions

The RME cell line RD (ATCC, Manassas, VA, USA) and the RMA cell line RH30 (DSMZ, Braunschweig, Germany) were routinely cultured in DMEM medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Biochrom, Berlin, Germany) and 1% l-glutamine (Biochrom, Berlin, Germany) in a humidified atmosphere containing 5% CO_2 at 37 C. Cell identity was proven by SLR analysis of the DNA profile using PowerPlex 16 (Promega, Mannheim, Germany). All cells were tested to be mycoplasma negative (Lonza, Cologne, Germany). The RMS cell lines were treated with the SGK1 inhibitor EMD638683 (APExBIO, Houston, Texas, USA, IC50 3μM) in the absence and presence of doxorubicin for the indicated periods and with the indicated concentrations. The concentration of EMD638683 has previously been shown to be effective [20, 33, 34].

RNA extraction and oligonucleotide microarrays

RMS tumor specimen and muscle tissue were immediately fixed in liquid nitrogen and stored at -80°C for further gene chip analysis. Total RNA extraction was carried out using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of the tumor RNA was monitored by an Agilent 2100 Bioanalyzer using the RNA6000 Nano LabChip Kit (Agilent Technologies, Boeblingen, Germany) as specified by the manufacturer. Samples with RIN>8 were considered for gene chip analysis. Affymetrix high-density oligonucleotide microarrays (GeneChip® Human Genome U133 Plus 2.0 Array, Affymetrix, Santa Clara, CA, USA) were used for gene expression analysis. Hybridization experiments and evaluation was done by the Microarray Facility Tuebingen, as originally described by Armeanu-Ebinger [35, 36]. The patient characteristics have been published by Armeanu-Ebinger et. al. [35] and were approved by the local ethics committees.

Real time PCR

Determination of SGK1 transcript levels was performed by RT-PCR. Total RNA was extracted from RD and RH30 cells using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of total RNA was performed using High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts, USA). Polymerase chain reaction (PCR) amplification of the respective genes were set up in a total volume of 20 µl using 40 ng of cDNA, 500 nM forward an reverse primer and 2x GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 58°C for 30 seconds and 72°C for 20 seconds. For the amplification the following primers were used (5`-3`orientaion):

SGK1 fw: GCTCCGACATAATATGCTTCTCC
SGK1 rev: GGCACTTCTAGCAAGACACAAGG;
TBP fw: GCC CGA AAC GCC GAA TAT
TBP rev: CCG TGG TTC GTG GCT CTC

Specificity of PCR product was confirmed by melting curve analysis. Real-time PCRs were performed on a CFX96 Real-Time System (Bio-Rad). All experiments were done in duplicates. Amplification of the house-keeping gene TBP (TATA binding protein) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the Δct method.

Cell viability assay

8 x 10^3 cells were seeded per well in 96-well plates. After overnight adherence of the cells and 72 hour treatment with EMD638683 (APExBIO, Houston, Texas, USA) in combination with and without doxorubicin cell viability was determined by means of a colorimetric MTT-assay measuring the reduction of tetrazolium salts to formazan derivatives by functional mitochondria. Lysis buffer (DMSO, SDS, acid) was added to solubilize the blue MTT-formazan product. The assays were performed in triplicates as previously described [37]. Absorbance was measured at 570nm.

Clonogenic assay

Cells were plated in 6-well plates at 500 cells per well in 2 ml of media. After attachment cells were treated with or without EMD638683 for 72 hours in a humidified atmosphere of 37°C and 5% CO2. After 72 hours cells were washed twice with PBS and fresh medium was added. The colonies grew 7-10 days, before being fixed with 80% methanol and stained with 0.2% crystal violet. Number of colonies (>50 cells) were counted microscopically [38].

In vitro migration assay

For transwell migration assays, 5×10^4 cells were placed in the top chamber with a non-coated membrane (24-well insert; 8 µm pore size, BD Biosciences) in serum-free medium whereas the lower chamber of the transwell system contained medium with 10% fetal bovine serum as chemoattractant. The cells were incubated during the experiment with EMD638683 for 24 h (RH30) or 48 h (RDs) in a humidified atmosphere of 37°C and 5% CO_2. Cells that did not migrate through the pores of the transwell membrane were removed by a cotton swab and washed with PBS. The transwells were moved to 4% paraformaldehyde (PFA) and incubated for 15 min at room temperature. Membranes were removed by scalpel, placed on slides and stained with Giemsa. The migrated cells bound on the lower surface to the membrane were then counted at 3 different regions using Axio Vision Release 4.8 software (Carl Zeiss Vision, Oberkochen, Germany).

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using unpaired Student *t*-test or ANOVA (Bonferroni test, Dunnett test) using GraphPad Prism version 4.0 (GraphPad Software, Inc.). Only results with $p<0.05$ were considered statistically significant.

Results

The present study addressed expression and functional significance of SGK1 in rhabdomyosarcoma cells. SGK1 transcription levels were determined in muscle tissue, alveolar (RMA) and embryonal (RME) rhabdomyosarcoma tumors. As illustrated in Fig. 1A, the SGK1 mRNA expression was significantly higher in alveolar rhabdomyosarcoma tissue

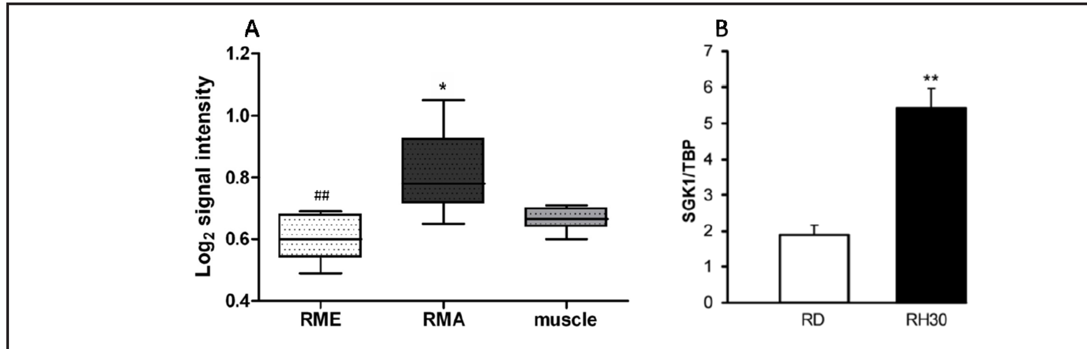

Fig. 1. SGK1 transcript levels in muscle tissue, rhabdomyosarcoma tumor tissue and cell lines. A. Individual Gene Chip® Human Genome U133 Plus 2.0 Array values and arithmetic means ± SEM of SGK1 transcript levels in muscle tissue (muscle, n=8), alveolar (RMA, n=5) and embryonic (RME, n=6) rhabdomyosarcoma tissue. *(p<0.05) indicates statistically significant difference from muscle, ##(p<0.01) indicates statistically significant difference from RMA. B. Arithmetic means ± SEM (n = 7) of SGK1 relative to TBP transcript levels in RD and RH30 cells. ** (p<0.01) indicates statistical significance

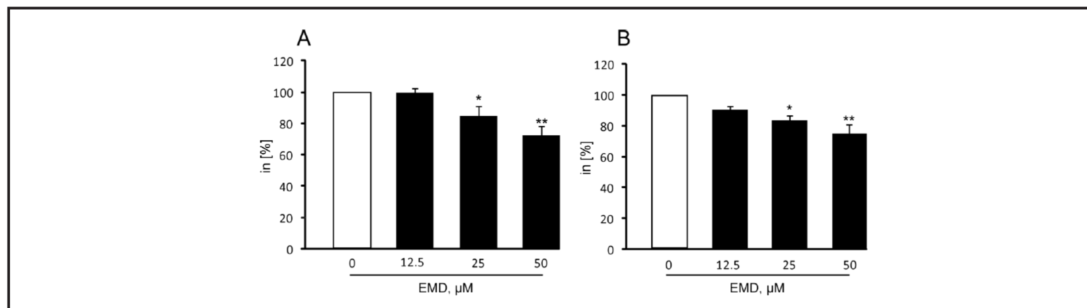

Fig. 2. Effect of the SGK1 inhibitor EMD638683 on the viability of RD and RH30 rhabdomyosarcoma cells. A,B. Arithmetic means ± SEM (n = 4) of the relative numbers of viable RD (A) and RH30 (B) cells following a 72 hours incubation in the presence of 12.5, 25 and 50 µM SGK1 inhibitor EMD638683 (black bars) relative to the absence of EMD638683 (white bars). * (p<0.05); ** (p<0.01) indicates statistical significance

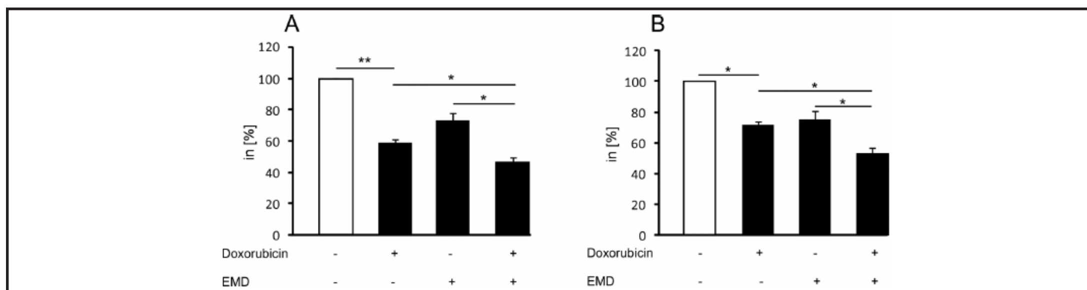

Fig. 3. Effect of the SGK1 inhibitor EMD638683 on doxorubicin sensitivity of RD and RH30 rhabdomyosarcoma cells. A,B. Arithmetic means ± SEM (n = 4) of the relative numbers of viable RD (A) and RH30 (B) cells following a 72 hours incubation in the absence (white bars) and presence (black bars) of 50 µM SGK1 inhibitor EMD638683 with and without 0.025 mg/ml doxorubicin. *(p<0.05), **(p<0.01) indicates statistical significance

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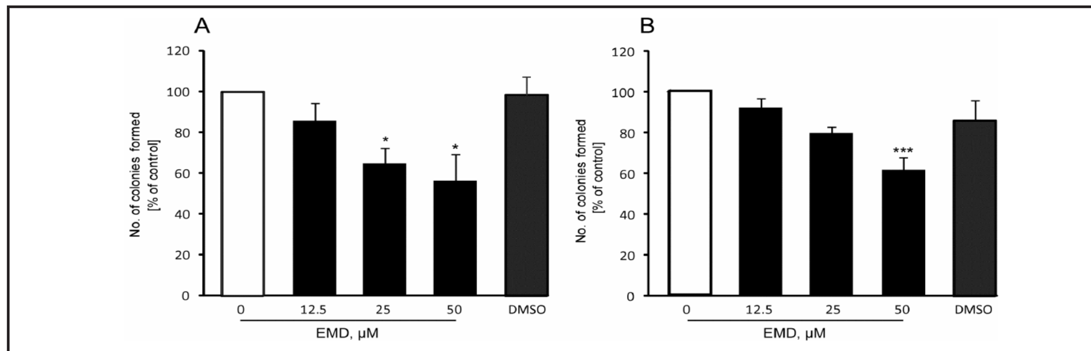

Fig. 4. Effect of the SGK1 inhibitor EMD638683 on cell proliferation in a clonogenic assay. A,B. Arithmetic means ± SEM (n =5) of the percentage of evolving clones of RD (A) and RH30 (B) cells following a 72 hours incubation in the presence of 12.5, 25 and 50 µM SGK1 inhibitor EMD638683 (black bars) relative to the clones in the absence of EMD638683 (white bars). * ($p<0.05$); *** ($p<0.001$) indicates statistical significance.

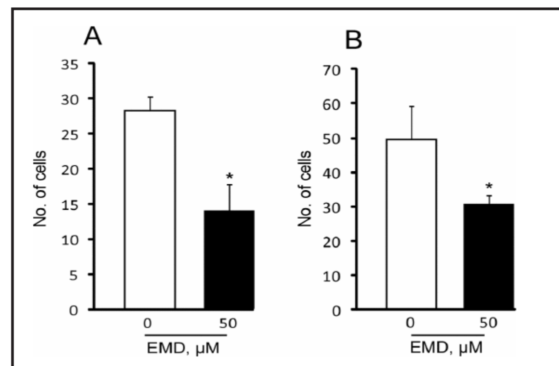

Fig. 5. Effect of the SGK1 inhibitor EMD638683 on the migration of rhabdomyosarcoma cells. A,B. Arithmetic means ± SEM (n = 6) of the percentage migrated RD (A) and RH30 (B) cells in the absence (white bars) and presence (black bars) of SGK1 inhibitor EMD638683 (50 µM, 24 h). *($p<0.05$) indicates statistical significance.

than in muscle tissue. The abundance of SGK1 transcript levels was significantly lower in embryonal than in alveolar rhabdomyosarcoma. SGK1 transcripts were further detected in the RD and RH30 rhabdomyosarcoma cell lines as shown in Fig. 1B – with significantly higher amounts of SGK1 transcripts present in RH30 compared to RD cells.

The specific and selective SGK1 inhibitor EMD638683, which is highly effective *in vivo* [30], was used to define the functional significance of SGK1 on survival, proliferation and migration of rhabdomyosarcoma cells. As illustrated in Fig. 2, EMD638683 treatment resulted in a statistically significant decline of cell viability in both RD and RH30 cells. A further series of experiments explored whether SGK1 modifies the negative effect of the cytotoxic drug doxorubicin with respect to cell viability. As shown in Fig. 3, doxorubicin treatment clearly decreased the viability of RD and RH30 cells, an effect that was significantly enhanced by the simultaneous administration of EMD638683.

The impact of SGK1 on clonal growth was quantified by colony forming assays. As shown in Fig. 4 - the relative numbers of evolving clones was significantly decreased in the presence of SGK1 inhibitor EMD638683 in RD and RH30 cells. A final series of experiments explored whether pharmacological SGK1 inhibition affected RD or RH30 cell migration. As shown in Fig. 5, the percentage of RD and RH30 cells that migrated through a transwell chamber was significantly decreased in the presence of EMD638683.

Discussion

In the present study we showed for the first time, that serum & glucocorticoid inducible kinase SGK1 is expressed in RMS, including both embryonal and alveolar rhabdomyosarcoma

tumor tissue samples and rhabdomyosarcoma cell lines RD and RH30. Interestingly, the more aggressive alveolar rhabdomyosarcoma express higher levels of SGK1 than the embryonal rhabdomyosarcoma. This observation is in full agreement with former observations that SGK1 interacts with several proteins like glycogen synthase kinase (GSK) 3 [39], N-myc downstream regulated 1 gene (NDRG1) [40, 41] and transcription factors FKRHL1 [42, 43] and NFκB [44] which in turn support cell survival, cell growth and inhibit apoptosis.

Importantly, pharmacological inhibition of SGK1 with the specific inhibitor EMD638683 was able to significantly decrease RMS cell`s viability, proliferation and capacity to migrate. Moreover, the sensitivity of RMS cell lines to the cytotoxic drug doxorubicin was enhanced, which is in complete accordance with reports that show SGK1 supporting cell proliferation, stimulating their migration and counteracting apoptosis in diverse tumor cell types [16, 29], including colorectal carcinoma [11-13, 16, 45], hepatocellular carcinoma [17, 18], breast cancer [18-20], prostate cancer [21, 22], glioblastoma [23-25], non-small cell lung cancer [26], meningiomas [27] and medulloblastoma [28].

SGK1 kinase is involved in the regulation of factors fundamental for activation, proliferation and cell survival including the up-regulation, abundance and activity of ion-channels and carriers as well as Na^+/K^+-ATPases. SGK1 also modulates the activity of enzymes belonging to important pathways such as glycogen-synthase-kinase-3 (GSK3), ubiquitin-ligase Nedd4-2, as well as the pivotal transcription factors NFκB and β-catenin [16]. SGK1 may also support and facilitate tumor growth by the activation of K^+ and Ca^{2+} channels, modulation of Na^+/H^+ exchanger and amino acid and glucose transporters; likewise via nuclear factor NFκB and β-catenin signaling while downregulating the transcription factors Foxo3a/FKHRL1 and p53 central players in cell survival [16]; SGK1 finally affects RAN/RANBP1/ RANGAP1 via SP1, thus playing a critical role in pre-miRNA nuclear export and epigenomic regulation [46].

Although this study did not focus on the pathways involved in the cyto reductive behavior of SGK1 towards RMS the observations of our present study indicates that inhibition of SGK1 may be a therapeutic option in rhabdomyosarcoma. It is noteworthy that the inhibitor is well tolerated *in vivo* [32], and that the phenotype of gene targeted mice lacking functional SGK1 is mild [31]. Thus SGK1 either alone or combined with doxorubicin presents as a promising strategy to prevent or overcome intrinsic mechanisms of resistance of RMS cells to chemo- and radiotherapy.

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Disclosure

The authors of this manuscript declare that they have no conflicts of interests

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