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Combined Treatment of ATRA with Epigenetic Drugs Increases Aggressiveness of Glioma Xenografts

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Abstract. *Background/Aim: Recently, anti-tumourigenic effects of all-trans-retinoic-acid (ATRA) on glioblastoma stem cells were demonstrated. Therefore we investigated if these beneficial effects could be enhanced by co-medication with epigenetic drugs such as the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) or the DNA-methyltransferase inhibitor 5-aza-2'deoxycytidine (5-AZA). Materials and Methods: Glioma stem cell xenografts were treated for 42 days with ATRA plus SAHA or ATRA plus 5-AZA or the correspondent monotherapies. Tumour sizes, histological features, proliferation and apoptosis rates were assessed. Results: Neither SAHA nor 5-AZA were able to enhance the anti-tumourigenic effect of ATRA. Instead, tumours became more aggressive. Combination of ATRA plus 5-AZA increased tumour size ($p<0.05$) and induced more frequent and larger necroses ($p<0.05$) and tumours were more invasive ($p<0.05$) in comparison to controls. A similar trend was observed for the combination of ATRA plus SAHA. Conclusion: Combining ATRA with epigenetic drug therapies led to the unwanted opposite effect and increased aggressiveness of glioma xenografts, arguing against future clinical applications of such combinations.*

Glioblastoma represents the most aggressive and most common type of brain tumour in adults (1). Even under maximum therapy, the average life expectancy is only about 15 months (2). A particular challenge in the treatment of high-grade gliomas are so called stem-like glioma cells (SLGCs) (3-6). This sub-population of cancer cells is endowed with properties of normal stem cells, such as self-renewal and pluripotency, increased tumourigenicity and resistance towards conventional therapies (3, 5). The eradication of SLGCs seems to be a crucial step in overcoming treatment resistance (7). In other cancer entities such as promyelocytic leukemia, the eradication of cancer stem cells has been successful by inducing their differentiation through the application of all-*trans*-retinoic-acid (ATRA) (8-10). However, in patients with glioma ATRA did not improve survival in initial clinical trials (11, 12), and neither the combination of ATRA with radiotherapy (13) nor with temozolomide (14) were successful. However, a sub-group of patients with glioblastoma did seem to benefit from this treatment (15, 16). Furthermore, all clinical trials so far have only included patients suffering from recurrent tumours. Therefore, it remains unclear if early therapy could be more successful. In line with this assumption, we recently demonstrated antitumourigenic effects of ATRA on glioblastoma stem-like cells *in vitro* and *in vivo* by treating tumour cells obtained from first-line resection (17). Although tumour growth was only delayed, it was not abolished and thus xenografted animals developed tumours with a penetrance of 100%. In order to increase the differentiation-inducing effect of ATRA, two epigenetic drugs have gained attention: the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA), which causes a general increase in histone acetylation; and the DNA methyltransferase inhibitor 5-azacytidine

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(5-AZA), which reduces the global methylation status of the DNA. Both combinations ATRA plus SAHA and ATRA plus 5-AZA have been successfully tested in preclinical models of other tumour entities (10, 18-20). Similar effects in gliomas are conceivable because locus-specific promoter methylation of tumour-suppressor genes seems to play an important role in glioma formation and progression (21-26). Therefore, re-expression of these genes through treatment with epigenetic drugs could help control tumour growth. Regarding SAHA, additional SAHA treatment augmented the effects of ATRA in acute promyelocytic leukemia and in prostate cancer both *in vitro* and *in vivo* (18, 19). Furthermore SAHA was able to enhance the pro-apoptotic effect of ATRA in medulloblastoma cells *in vitro* (20). In addition, medulloblastoma xenografts treated with the same combination developed smaller tumours, while it was also possible to reduce the dose and thus toxicity of SAHA by 40% and no accumulation of toxic side-effects was observed (20). To evaluate if these types of combination therapies exert similar beneficial effects in glioblastoma, we studied the effectiveness of ATRA plus 5-AZA and ATRA plus SAHA in SLGC tumours.

Materials and Methods

Preparation of cells for stereotactic implantation. The well-characterized human SLGC line NCH644 was grown as described and used for xenotransplantation assays (17, 27). Origin of the cells was confirmed by short tandem repeat profiling as compared to the original patient tissue. NCH644 spheroids were sedimented. After assessing cell density (Casy Counter, Schärfe Systems, Reutlingen) cells were transferred into a new Falcon tube and centrifuged at 300 × *g*. The pellet was re-solved in phosphate-buffered saline and the cell density was adjusted to 2×10^6 cells/ml. A total of 10^4 cells in 5 μl were used for implantation.

Study design. Six-week-old female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (n=62) (Charles River, Sulzfeld, Germany) were housed under specific pathogen-free conditions. All animal experiments were carried out according to the German Animal Protection Law (Regierungspräsidium Karlsruhe, Germany; approval number 35-9185.81/G-128/12). Institutional guidelines for animal welfare and experimental conduct were followed. Cells were implanted stereotactically into the right hemisphere of each mouse as described elsewhere (17). Subsequently, mice were randomized into five treatment groups (ATRA (n=10), SAHA (n=10), 5-AZA (n=10), ATRA plus SAHA (n=10), ATRA plus 5-AZA (n=10)) and a control group (n=12). Medication was administered intraperitoneally every third day starting on the day of implantation. Controls received vehicles only. ATRA was dissolved in purified corn oil, SAHA in 2-hydroxypropyl-b-cyclodextrin and 5-AZA in DMSO diluted 1:40 with saline. All mice received equal amounts of vehicle simultaneously. ATRA, SAHA and 5-AZA (Sigma–Aldrich, Munich, Germany) were administered at a dose of 0.2 mg/injection day, 1.25 mg/injection day and 0.0025 mg/injection day, respectively. At day 42, mice were sacrificed and the brains were removed and cryopreserved for further analyses.

Assessment of tumour volume. To determine tumour volume, each tumour-containing brain tissue was cut in coronary sections over the entire tumour length and stained with haematoxylin and eosin (H&E). The tumour area was measured every 30th section (≅150 μm) over the entire tumour extension with Cell Imaging Software (Olympus, Hamburg, Germany). The total tumour volume was approximated by overlaying all tumour-bearing sections as $V_{Tumour} \approx \Sigma((a_i + a_i{+}1)/2 \times h)$, where ai is the area of the tumour of the section "*i*", *i* is the number of the section from 1 to n, and h distance of the sections a_i and a_i+1.

Assessment of histological changes. To assess the extent of necrosis and infiltration, the following scoring system was applied. Necroses were scored as follows: no necrosis on any of the sections=0, small necroses on fewer than 30% of the sections=1, small to middle-sized necroses in 30-90% of the sections or with sporadic extended necrotic areas=2, large necroses on >90% of the sections=3. Likewise, we assessed tumour infiltration: 1=weak invasion, relatively smooth limited tumour margins in all sections, no or very few stronger infiltration zones, no multifocal tumours; 2=circumscribed but multifocal tumours or strong infiltration zones in some sections; 3=multifocal tumours with strong infiltration zones on almost all sections.

Immunohistochemistry. Staining of representative mouse tissue sections of all treatment groups was carried out as described elsewhere (28). Primary antibodies used were mouse anti-human nuclei (1:100), mouse anti-human Ki-67 (1:25) (both BD Pharmingen, Hamburg, Germany); rabbit anti-human acetyl-H2A (1:25), rabbit anti-human acetyl-H2B (1:25), rabbit anti-human acetyl-H3 (1:25), rabbit anti-human acetyl-H4 (1:100; all Cell Signaling Technology, Frankfurt am Main, Germany). Histochemical detection of apoptosis was performed based on the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. TUNEL staining evaluation was carried out on representative sections of tumours of all treatment groups. Necrotic areas were excluded from the analyses to avoid false-positive staining results. TUNEL-positive cells were counted per visual field on three hotspots at a 20-fold magnification.

MassARRAY. The Epityper®-MassARRAY Essay of Sequenom® represents a valid high-throughput method for quantitative DNA methylation analysis (29). Tumour tissues of the control group and the two groups treated with 5-AZA were tested to assess changes in DNA methylation. To ensure that the transcriptome was not affected by varying numbers of normal cells, tumor areas were dissected selectively by laser capture microdissection. Genomic DNA was extracted and analyzed for integrity using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then DNA was bisulfite-converted using the EZ-DNA-Methylation-Kit (Zymo Research, Irvine, CA, USA). MassARRAY was carried out subsequently using specific long interspersed nuclear element 1 (*LINE1*) primers (30). The methylation state of the amplicons was finally determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Statistical methods. Distribution of tumour volumes and histological parameters were compared using Mann–Whitney *U*-test. Treatment groups were tested against the control group and the two combination therapy groups were additionally tested against the ATRA

monotherapy group. Comparison of the methylation status between 5-AZA-treated and untreated mouse tissues was carried out using a two-sided *t*-test. *p*-Values of 0.05 or less were considered significant.

Results

Antitumourigenic effects of ATRA are not enhanced by 5-AZA or SAHA co-medication. To study the additional antitumourigenic effect of combining ATRA with 5-AZA, and with SAHA, mice bearing xenografts were assigned to six treatment groups including the respective monotherapies, combinatorial treatments and vehicle-treated controls. All mice developed tumours during our observation period (42 days), endowed with features of high-grade glioma, such as increased proliferation (Figure 1). Interestingly, mitotic activity seemed to be lowered only in the ATRA and SAHA monotherapy groups. *5-AZA* and *SAHA* reach the tumour site at therapeutic concentrations. Since combination therapies did not have the anticipated enhanced antitumour effect, we questioned if 5-AZA and SAHA reached the tumour site at therapeutic concentrations. Regarding 5-AZA, we determined the non long terminal repeat retrotransposon 1 (*LINE1*) methylation status compared to controls by MassARRAY. For SAHA, we analyzed acetylation changes of histones H2B, H3 and H4 in all SAHA-treated tumours by immunohistochemistry. *LINE1* methylation decreased significantly, on average by 14.1%, in 5-AZA-treated animals (*t*-test: $p=0.012$, Figure 2A). Yang *et al*. showed in previous work that at demethylation of about 15% induced by the 5-AZA-derivative decitabine, a plateau was reached, and the demethylating effect did not increase with higher doses of medication (30). Since *LINE1* represents 17% of the human genome (31), the demethylation measured in the present study is a strong indicator that 5-AZA passed the blood–brain barrier and changed the overall methylation in the tumour tissue successfully.

In all SAHA-treated mice, including those treated with monotherapy, as well as the combination groups, we found an increased acetylation of histones H2B, H3 and H4 compared to non-SAHA-treated tumours (Figure 2B). We saw a distinct and rather homogenous staining of tumour cells in both treatment groups. Of note, occasionally a few unstained cells were found adjacent to well-stained cells (Figure 2B, black arrows). These effects were particularly pronounced in the two mice in which the tumours reached high tumour volumes and thus might represent outliers from the group.

ATRA combination therapies increase tumour size. Finally, we quantified tumour volumes in the different treatment groups (Figure 3). In line with the previously described anti-tumourigenic effects of ATRA (17), the smallest median tumour volume was observed in animals treated with ATRA alone, followed by those treated with SAHA and 5-AZA monotherapy. In contrast, median tumour volumes in ATRA combination treatment groups were 8.7- and 8-fold higher when applying SAHA and 5-AZA in addition to ATRA compared to ATRA medication alone. In the ATRA plus 5-AZA treatment group, the increase in tumour sizes were significant compared to those under ATRA monotherapy, while for the ATRA plus SAHA therapy, a trend towards larger tumours remained, most probably due to the observed outliers.

Apoptotic cells are reduced after ATRA combination therapies. In order to determine whether the combination of ATRA plus SAHA or ATRA plus 5-AZA increased the number of apoptotic cells in the tumour tissue, TUNEL staining on at least five representative tumours of each group was performed. The highest frequencies of apoptotic cells were recorded after ATRA, treatment followed by 5-AZA and SAHA monotherapies. Again, both combination treatments revealed the worst effect. Particularly in the ATRA plus SAHA combination group, the number of TUNEL-positive cells was even lower than in the controls (Figure 4).

Combination treatments result in more invasive and necrotic tumours. Next we questioned the influence of medication on tumour properties such as invasiveness and the occurrence of necrosis. H&E-stained sections of all tumours were reviewed regarding the extent of invasion and necrosis (Figure 5). In line with smaller tumour volumes and increased numbers of apoptotic cells, we found significantly less frequent and smaller necroses ($p=0.027$) in tumours treated with ATRA monotherapy in comparison to controls.

Likewise, we saw a trend in the SAHA monotherapy group towards smaller and less frequent necrotic areas ($p=0.0685$), while tumours of 5-AZA-treated animals resembled control tumours. Corresponding to the trend towards higher tumour volumes in the combination treatment groups, we found more frequent and more extended necroses in the ATRA plus SAHA and the ATRA plus 5-AZA treatment groups ($p\leq0.05$). This effect was even more pronounced when comparing tumours of ATRA-treated animals with those treated with ATRA plus SAHA ($p=0.0017$ and ATRA plus 5-AZA ($p=0.001$), respectively. Similar paradoxical effects were observed when looking for the extent of invasion in tumours of the different treatment groups. Lowest invasion scores were recorded in tumours from ATRA-treated mice, while the strongest, significantly enhanced invasion was found in those from ATRA plus 5-AZA treated mice, followed by those treated with co-medication of ATRA with SAHA.

Discussion

Although numerous clinical studies in the late 1990s identified only moderate therapeutic effects of ATRA as a single agent in patients with glioblastoma (11, 32, 33), we recently demonstrated that ATRA reduces the CD133-positive cell pool

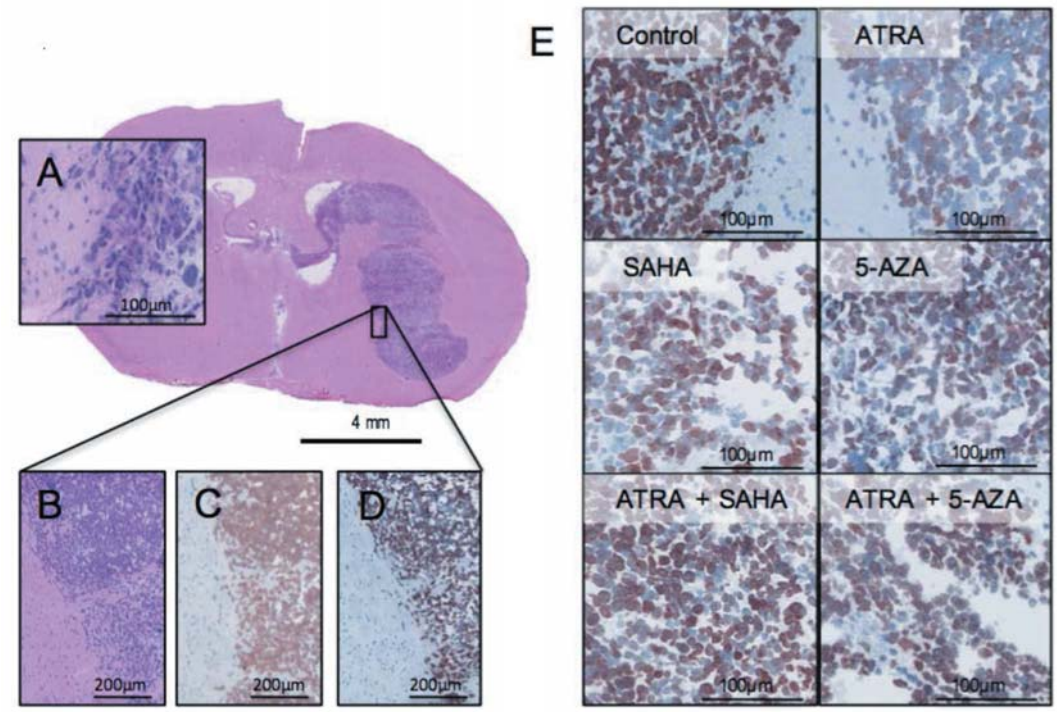

Figure 1. *Proliferation of NCH644-derived tumours in different treatment groups. Representative haematoxylin and eosin (H&E) staining at low (A) and (B) higher magnification of a tumour-bearing mouse brain in control group. C: Anti-human nuclei staining confirmed the human origin of tumour cells and allowed a clear discrimination between xenotransplanted and host cells. Ki-67 staining of untreated tumours (D) and in representative tumours from mice under different treatment conditions (E).*

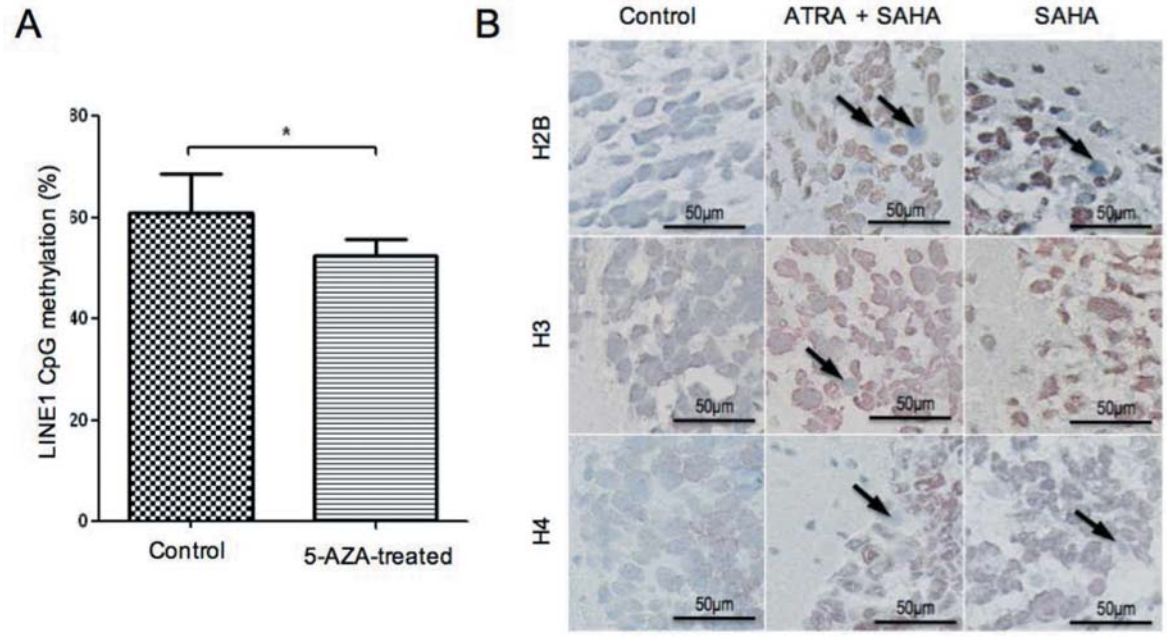

Figure 2. *Changes in methylation and acetylation upon 5-aza-2'deoxycytidine (5-AZA) and suberoylanilide hydroxamic acid (SAHA) treatment A: Global methylation of representative long interspersed nuclear element 1 (LINE1) cytosine-phosphate-guanine dinucleotides (CpGs). Columns show the mean percentage methylation of representative LINE1 CpG islands of tumours from AZA-treated vs. control mice. *p≤0.01. B: While staining with antibodies against acetylated histones H2B, H3 and H4 in the control group only led to faint staining of the tumour tissue, which barely contrasted against the non-specific staining of the surrounding mouse tissue, distinct rather homogenous staining of tumour cells was apparent in tumours from mice of both treatment groups. Few individual cells remained unstained (black arrows).*

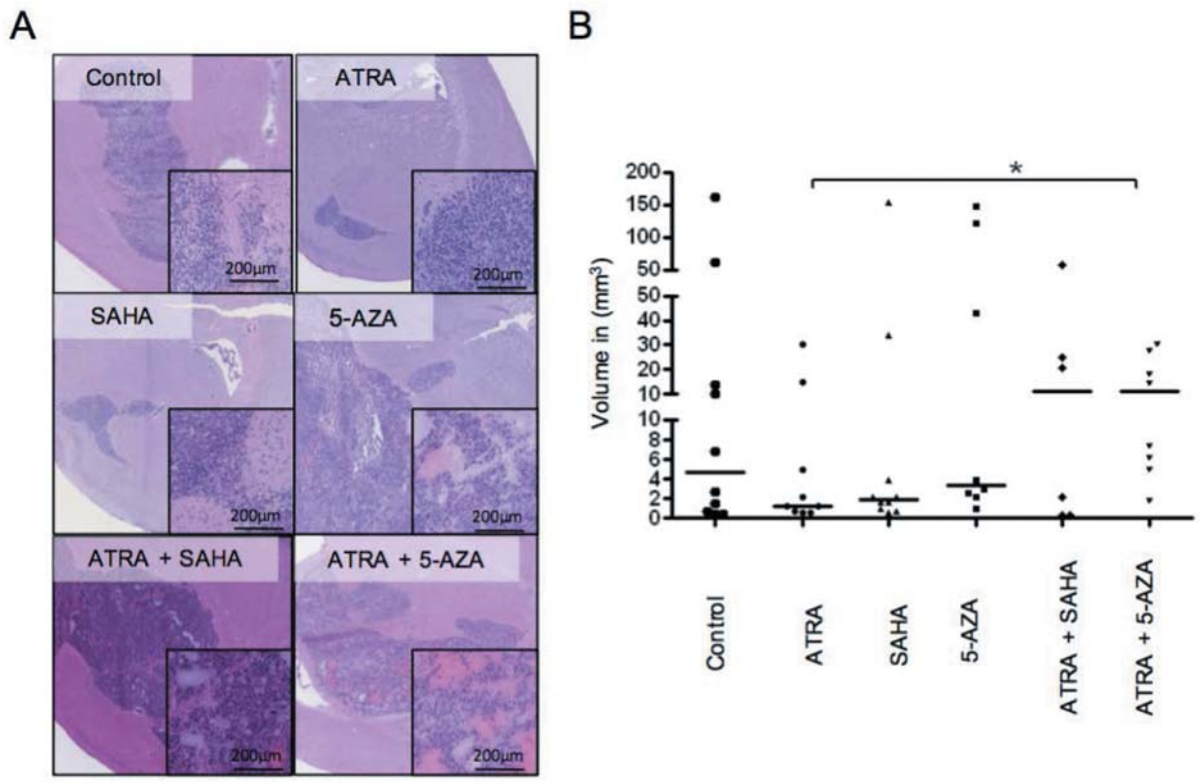

Figure 3. *Growth and sizes of NCH644-derived tumours from mice of different treatment groups. A: Representative haematoxylin and eosin (H&E) staining. B: Tumour volumes according to group assignment (*p≤0.05).*

and exerts anti-tumourigenic effects on SLGCs (17). Therefore in the present study, we attempted to enhance these anti-tumourigenic effects of ATRA by co-medication with well-known epigenetic drugs of two different classes: the HDAC inhibitor SAHA and the DNA-methyltransferase inhibitor 5-AZA. Although confirming the anti-tumourigenic effects of ATRA monotherapy described by Campos and co-workers (17), the combination of ATRA with both SAHA and 5-AZA resulted in the opposite effect and increased the aggressiveness of the tumours substantially, including their tumour size, frequency and extent of necroses and invasiveness, and reduced apoptosis.

We selected the HDAC inhibitor SAHA because numerous reports described beneficial effects in a variety of tumour entities ranging from a disruption of the cell cycle and the production of DNA damage to a disturbance of the mitotic activity, the induction of apoptosis and the inhibition of neoangiogenesis (34). Moreover, SAHA increased the pro-apoptotic effect of ATRA on medulloblastoma cells *in vitro* and markedly reduced tumour size and growth of xenografts (20). For serum-cultivated glioma cells treated with SAHA (35) and for prostate cancer cells treated with the combination of ATRA and SAHA (19), reduced tumour cell proliferation is described. Unfortunately, in the present study, we did not observe any of these synergistic effects in SLGC tumours. While in both the ATRA and SAHA monotherapy groups, histopathological tumour parameters associated with malignancy were less pronounced, treatment with a combination of both substances seemed to entail an aggravation of the disease. However, since we unequivocally demonstrated histone acetylation in SAHA-treated tumours, we can exclude insufficient bioavailability. Future investigation is needed to clarify if these unwanted therapeutic effects are due to the fact that we tested the medication against highly aggressive SLGC cells instead of using serum-cultivated glioma cells, which are assumed to be less representative and less aggressive (36).

The DNA-methyltransferase inhibitor 5-AZA was the second agent whose additional impact on the effect of ATRA we investigated in the present study. In recent years, a number of cell-cycle controlling genes have been shown to be down-regulated in glioblastomas by locus-specific promoter methylation (21-24, 26, 37, 38). Moreover, it is known that global de-methylation during 5-AZA therapy is well correlated with a decrease of methylation at individual promoter regions (30). Consistent with this, we demonstrated that the cellular

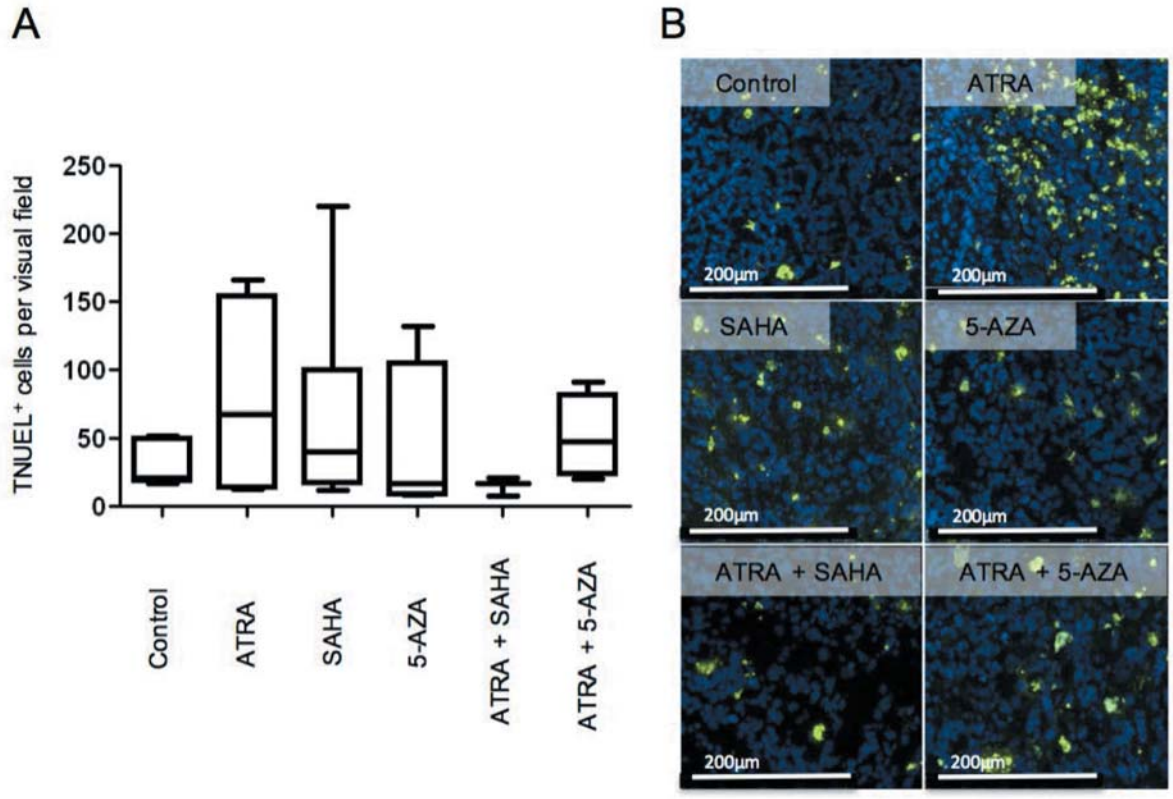

Figure 4. *Extent of apoptosis in NCH644-derived tumours from mice of different treatment groups. A: Number of terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive (TUNEL⁺) cells per visual field. Box and whisker plot of means of at least three hotspots/per tumour of at least three tumours per group. Horizontal bars represent the median, the box represents two quartiles±the minimum and maximum rate of apoptosis (whisker). B: Representative images of TUNEL-stained tumours.*

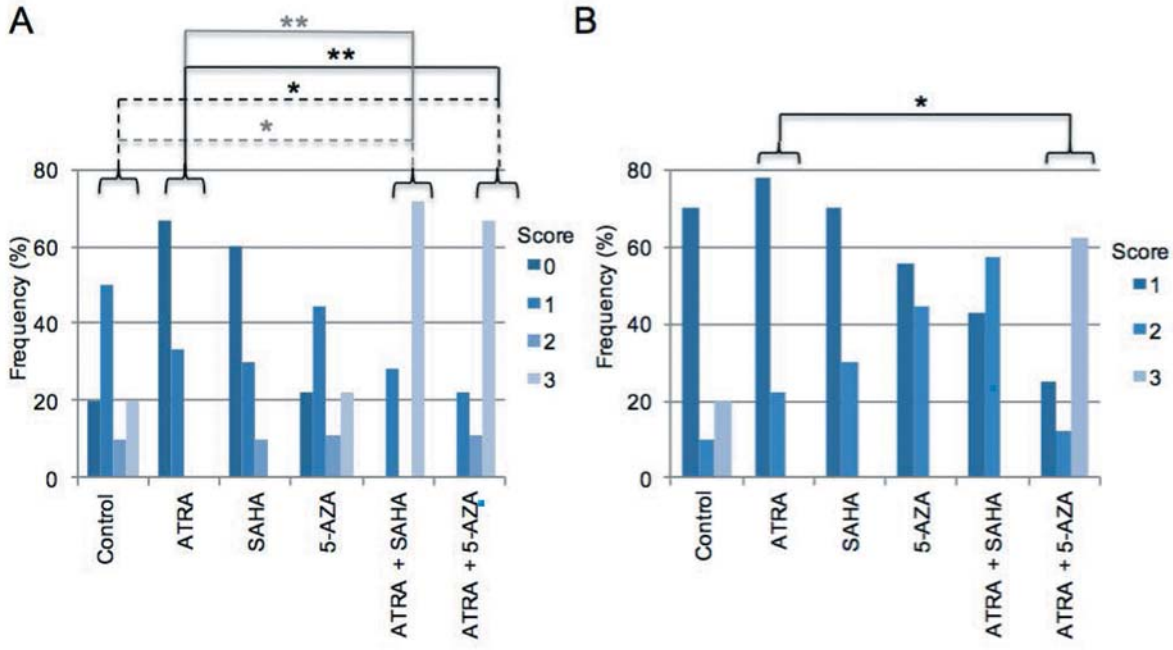

Figure 5. *Frequency and extent of necrosis (A) and invasion (B) of NCH644-derived tumours from mice of different treatment groups. Columns: number of tumours assigned to each score as a percentage. *p≤0.05; **p≤0.01.*

retinoic acidbinding protein 2 (CRABP2), an intracellular transport protein, essential for shuttling ATRA into the nucleus and thus for its differentiation-inducing effect, is up-regulated upon 5-AZA treatment (39). Moreover, Fu and coworkers have shown in medulloblastoma that the *CRABP2* promoter region is frequently hypermethylated in ATRA-resistant cells (40). Additionally, frequent hypermethylation of the retinoic acid receptor β (*RARβ*) promoter region in ATRA-resistant head and neck squamous cell carcinoma cells has been described (41). The latter was reduced upon treatment of head and neck squamous cell carcinoma cells with 5-AZA (42). It therefore seems reasonable to expect that 5-AZA treatment should augment the antitumourigenic effect of ATRA on SLGCs as it did in other tumour entities (10). However, as in the case of SAHA, treatment with this combination increased the tumourigenicity of SLGCs with regard to tumour size and histological features. Again, insufficient bioavailability of 5-AZA can be excluded based on our results for *LINE1* demethylation. However, it is in turn conceivable that the biology of highly aggressive SLGCs is not susceptible to epigenetic treatment. In accordance with this assumption, it was recently shown that promoter hypermethylation in gliomas caused by isocitrate dehydrogenase 1 (*IDH1*) mutations results in significantly improved survival of the respective patients (43). Thus, co-medication with 5-AZA also does not seem appropriate for increasing the antitumour effects of ATRA. In contrast, our results suggest that the aggressiveness of glioblastoma stem cells was enhanced by this combination.

In summary, we are forced to state that despite promising antitumour effects being described for all three single agents ATRA, SAHA and 5-AZA, our results raise the question if their combined application might not only be ineffective but also even harmful for patients with glioblastoma. Further *in vitro* and *in vivo* studies will be needed to identify the underlying molecular mechanisms before using such treatment combinations in clinical trials on this tumour type.

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