Original Article

Trichostatin A Inhibits Rhabdomyosarcoma Proliferation and Induces Differentiation through MyomiR Reactivation

(rhabdomyosarcoma / TsA / muscle differentiation / myomiR / miRNA)

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Abstract. Rhabdomyosarcoma (RMS) is a malignant tumour of soft tissues, occurring mainly in children and young adults. RMS cells derive from muscle cells, which due to mutations and epigenetic modifications have lost their ability to differentiate. Epigenetic modifications regulate expression of genes responsible for cell proliferation, maturation, differentiation and apoptosis. HDAC inhibitors suppress histone acetylation; therefore, they are a promising tool used in cancer therapy. Trichostatin A (TsA) is a pan-inhibitor of HDAC. In our study, we investigated the effect of TsA on RMS cell biology. Our findings strongly suggest that TsA inhibits RMS cell proliferation, induces cell apoptosis, and reactivates tumour cell differentiation. TsA up-regulates miR-27b expression, which is involved in the process of myogenesis. Moreover, TsA increases susceptibility of RMS cells to routinely used chemotherapeutics. In conclusion, TsA exhibits anti-cancer properties, triggers differentiation, and thereby can complement an existing spectrum of chemotherapeutics used in RMS therapy.

Introduction

Musculoskeletal sarcomas are relatively rare, aggressive malignancies of bone and soft tissues, often affecting children and young adults with devastating consequences in terms of both morbidity and mortality.

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children, accounting for 5 % to 10 % of all paediatric malignancies. It is an aggressive form of sarcoma that in the vast majority of cases occurs in children younger than 18 years, and despite being a rare cancer, it accounts for approximately 40 % of all soft tissue sarcomas in children. There are two main histological subtypes, alveolar (ARMS) and embryonal (ERMS). The majority of ARMS are associated with characteristic fusion genes, either \( \text{PAX3-FOXO1} \) or, less frequently, \( \text{PAX7-FOXO1} \) and rarer variants. Patients with \( \text{PAX3-FOXO1} \) fusion gene-positive tumours are generally considered to have a poorer prognosis (Pappo, 1995; Collins et al., 2001; Ognjanovic et al., 2009; Wang, 2012).

RMS resembles developing skeletal muscle that expresses multiple genes characteristic of muscle cell differentiation (like myogenin and myogenic differentiation, MyoD) but fails to complete the differentiation programme. Expression of skeletal muscle-specific markers suggests commitment to the skeletal muscle lineage; however, PAX3-FOXO1 has been shown to suppress transcriptional activity of MyoD target genes, and direct downstream targets of the fusion gene may also be involved in suppressing differentiation (Altmannsberger et al., 1985; Tonin et al., 1991; Parham, 2001; Sebire and Malone, 2003; Kaspar et al., 2015).

DNA methylation and histone modifications constitute major mechanisms that are responsible for epigenetic regulation of gene expression during development and differentiation. Both ARMS and ERMS are characterized by important epigenetic mutations and an epigenetic imbalance that may affect some important genes. One of the most common is loss of imprinting (LOI) at chromosome locus 11p15.5 that alters expression of the tandem genes \( \text{IGF2} \) and \( \text{H19} \) (Zhan et al., 1994; Anderson et al., 1999). Other epigenetic changes involving aberrant DNA methylation (i.e., hypermethylation of gene promoters involved in development and differentiation – \( \text{DNAJA4, IRX1, ALX3} \)), histone H3 methylation or histone acetylation resulting in transcriptional repression (Polycomb Group (PcG) protein YY1, enhancer of zeste homolog 2) leading to differentiation blockage have also been identified (Chen et al., 1998; Gastaldi et al., 2006; Goldstein et al., 2007; Chase and Cross, 2011; Mahoney et al., 2012; Marchesi et al., 2014; Shern et al., 2015).
Histone deacetylase (HDAC) inhibitors are natural or synthetic small molecules that interfere with the function of HDACs. They are emerging as potent anticaner agents as a result of their effective anti-proliferative activity in a wide variety of tumours, mediated by mitotic defects through aberrant acetylation of histone and non-histone proteins. It has been reported that trichostatin A (TsA), a potent specific inhibitor of HDAC, is able to lead to cell growth arrest, differentiation and/or apoptosis in a number of cancers and disease states (Haberland et al., 2009). What is more, it has been shown that inhibition of HDAC activity may affect expression of microRNAs (miRNAs). They may trigger re-expression of tumour suppressor miRNAs and repression of oncogenic miRNAs (Ali et al., 2015).

miRNAs are a class of non-coding RNA molecules that are differentially expressed in normal tissues compared to cancers and influence expression of their target genes by gene silencing. By imperfect complementarity and hybridization with the target mRNA, these small molecules of 19–24 nucleotides cause either mRNA molecule degradation or translational inhibition. miRNAs may act as potential therapeutic targets as they influence cell proliferation, apoptosis and differentiation. Restoration of normal miRNA levels has been demonstrated to decrease cancer cell growth and progression (Clape et al., 2009; Aslam et al., 2012; Ali et al., 2015). In RMS, a group of particular miRNAs is of special interest. MyomiRs are muscle-specific miRNAs (miR-1, miR-133, miR-206, miR-208, miR-208b and miR-499) that have been identified and shown to be involved in a range of processes including myogenesis (proliferation, differentiation and fibre type specification), regeneration, hypertrophy and muscular dystrophy (McCarthy, 2008; McCarthy et al., 2009; Rota et al., 2011). Studies on fibro-adipogenic progenitors isolated from murine dystrophic muscles indicate a potent regulatory network linking HDAC with myomiRs. HDAC inhibitor (TsA) is able to restore dormant myogenic programme by derepression of miR-1, miR-133, miR-206. Further activity of the reactivated myomiRs leads to promyogenic differentiation (Cacchiarelli et al., 2010; Saccone et al., 2014).

Despite the improvements that have been made in the treatment of solid tumours in recent years, there is an immediate and important need to develop novel therapeutic options. Re-differentiation of RMS to its original skeletal muscle tissue type is a novel strategy to inhibit tumour progression. In this work, we aimed to investigate the possible role of TsA – a potent pan-family HDAC inhibitor – in the induction of terminal differentiation of RMS cells. We show that the HDAC inhibitor (TsA) may reactivate multiple downregulated myomiRs that may serve as other potential therapeutic targets in the treatment of RMS.

Material and Methods

Cell lines

In this study, two human rhabdomyosarcoma cell lines were used: RH30 (type ARMS) and RD (type ERMS). The cells were cultured in RPMI-1640 (RH30) and DMEM (RD) medium (Sigma-Aldrich, St. Louis, MO), supplemented with penicillin (100 IU/ml), streptomycin (10 μg/ml) (Life Technologies, Grand Island, NY) and 10% heat-inactivated foetal bovine serum (FBS) (Life Technologies). The cells were cultured at 37 °C in 5% CO₂ at an initial cell density of 2.5 × 10⁴ cells/flask (Corning Inc., Corning, NY). Medium was changed every 48 h.

Cell proliferation

The RH30 and RD cells were plated on a 24-well plate at an initial density of 1 × 10⁵ per well in the presence or absence of TsA (75, 150, 300, 500, 750, 1000, 2000 nM) (Sigma-Aldrich), and chemotherapeutics: vincristine (10, 50, 100, 200, 500, 800, 1000 pg), actinomycin D (1, 2, 5, 10, 20, 50, 100 nM), cyclophosphamide (25, 50, 200, 600, 750, 950, 1100 nM) (Sigma-Aldrich). The number of cells was calculated at the time points: 24, 48 and 72 h. At each time point, the cells were trypsinized in order to count them using a Navios flow cytometer (Beckman Coulter, Inc. Indianapolis, IN).

Apoptosis assay

Apoptosis was detected using annexin V and propidium iodide (PI) (BD Biosciences, San Jose, CA) and evaluated by flow cytometry. The cells were plated on a 6-well plate at an initial density of 0.5 × 10⁶ per well. After 24 h, different TsA concentrations (75, 300, 500, 1000 nM) were added to the cells. RMS cells were washed with PBS and stained with annexin V-FITC (5 μl) and PI (5 μl) in 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM CaCl₂) in the dark for 15 min at room temperature. Early apoptotic cells were annexin V+ PI–, late apoptotic populations or secondary necrotic cells were annexin V– PI+.

Viability test

Cell viability was evaluated using the Cell Proliferation Reagent WST-1 assay (Sigma-Aldrich). RMS cells were seeded in a 96-well plate at the density of 2 × 10⁴ cells/well. The cells were cultured in 100 μl medium. After 24 h, respective TsA concentrations (75, 150, 300, 500, 750, 1000nM) were added to the wells. After 48 h, 10 μl WST-1 reagent was added and cells were incubated for 30 min. Afterwards, absorbance was measured using a spectrophotometric microplate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland) at 450 nm (with 620 nm background correction). Results were normalized to the control, non-stimulated cells. Calculation of cell viability: number of viable cells = [(test-blank)/(control-blank)] × 100 %. The experiment was repeated three times in triplicates.
Immunohistochemical staining

Cells cultured on cover glasses were fixed with 4% formaldehyde solution (Sigma-Aldrich, Poznan, Poland); after washing in PBS, cells were permeabized in 0.5% TRITON X-100 solution in PBS (Sigma-Aldrich) for 10 min. After double wash in PBS, cells were incubated with blocking serum (2.5% horse serum, Vector Laboratories, Burlingame, CA) for 20 min. Next, the cells were incubated with mouse anti-human myosin HC primary antibody (R&D Systems, Minneapolis, MN) for one hour at room temperature (RT). After incubation, cells were washed twice and incubated with FITC-conjugated anti-mouse secondary antibody (Sigma-Aldrich) for one hour at RT. Next, after washing in PBS, nuclei were counterstained with DAPI (Sigma-Aldrich) and samples were mounted in fluorescent mounting medium (Dako, Glostrup, Denmark).

Images were captured in an FV1000 confocal system combined with an Olympus IX81 inverted microscope (Olympus, Hamburg, Germany). Images were collected in two separate channels for DAPI and FITC and shown as merged channels. Microphotographs were scanned with an ×40 objective, scale bar 30 μm.

miRNA real-time quantitative reverse transcription PCR (RQ-PCR) analysis

Quantitative RT-PCR to validate selected miRs was performed using a PerfeCTa microRNA assay kit (QuantaBio, Beverly, MA) and corresponding primers (Oligo.pl, Poznan, Poland), according to manufacturer’s instructions. Sequences of the miRNA oligonucleotides were obtained from the miRBase Release 21 (www.mirbase.org). Five μl of eluted RNA was polyadenylated and reverse-transcribed to cDNA, followed by real-time PCR analysis using PerfeCTa Universal PCR Primer and SYBR Green (Applied Biosystems, Foster City, CA) in a total reaction volume of 20 μl. Primers were added at a final concentration of 0.2 μM. SNORD44 and U6 expression levels were used to correct for cDNA input, with the Human Positive Control Primer (Quanta BioSciences, Manassas, VA). Data were analysed by normalizing miR Ct values to the positive control Ct value to obtain ΔCt values.

mRNA real-time quantitative reverse transcription PCR (RQ-PCR) analysis

Total RNA was isolated from cells treated with TsA and from cell controls with an RNaseasy Kit (Hilden, Germany). The RNA was reverse-transcribed with MultiScribe reverse transcriptase and oligo-dT primers (Applied Biosystems). Quantitative assessment of mRNA levels was performed by real-time RT-PCR (RQ-PCR) in an ABI 7500 instrument with Power SYBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95 °C (15 s), 40 cycles at 95 °C (15 s), and 60 °C (1 min). According to melting point analysis, only one polymerase chain reaction (PCR) product was amplified under these conditions. The relative quantity of a target, normalized to the endogenous control β-2 microglobulin gene and relative to a calibrator, is expressed as 2^(-ΔCt) (fold difference), where Ct is the threshold cycle, ΔCt = (Ct of target genes) – (Ct of endogenous control gene, β-2 microglobulin), and ΔΔCt = (ΔCt of samples for target gene) – (ΔCt of calibrator for the target gene). The following primer pairs were used: CKM F: 5'- GGC ATC TGG CAC AAT GAC AA – 3'; CKM R: 5'– GAT GAC CCG GAG GTG ATC CT – 3'; MYH1 F: 5'- GGG AGA CCT AAA ATT GGC TCA A – 3'; MYH1 R: 5'- TTG CAG ACC GCT CAT TTC AAA – 3'; MYH2 F: 5'- AAG GTC GGC AAT GAG TAT GTC A – 3'; MYH2 R: 5'- CAA CCA TCC ACA GGA ACA A CT TCT TC – 3'; MYL1 F: 5'- TGC TGA ACT CCG CCA TGT T – 3'; MYL1 R: 5'- CCA TCA GGG CTT CCA CT TCT CT – 3'.

miRNA transfection

Enhanced expression of miR-27b was performed by transfecting RD and RH30 cells with miR-27b-5p and miR-27b-3p mimics (Sigma-Aldrich). Cells were seeded into 24-well plates (5 × 10^4) and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 40 μM miRNA for 24 h. In parallel, cells were transfected with MISSION miRNA Negative Control (Sigma-Aldrich). Next, cells were subjected to expression analysis by RQ-PCR.

Results

In our study, we aimed to assess the effect of trichostatin A – histone deacetylase pan-inhibitor – on the biology of two rhabdomyosarcoma cell lines – ARMS (RH30) and ERMS (RD). In the first approach, we evaluated the influence of TsA on cell viability by using the cell proliferation reagent WST-1 assay (Fig. 1). WST-1 is a stable tetrazolium salt that is degraded by metabolically active cells. Use of this assay allowed us to define the IC50 value for TsA concentrations for both tested cell lines during 48 h treatment. They were 484 nM and 531 nM for RH30 and RD, respectively. In Fig. 2, we evaluated the effect of different doses of TsA (from 75 nM to 1 μM) on the two tested cell lines. In both cases (RD and RH30) we noted abrogation of cell proliferation within 72 h of experimentation.

As shown in Fig. 3, by employing PI staining and annexin-V binding, we showed that increasing doses of TsA (≥ 500 nM range) induced apoptosis in RMS cell lines (Fig. 3). Thus, we may speculate that at lower doses (~500 nM), TsA acts as a potent cytostatic, and at higher doses, it triggers the apoptotic response leading to cell depletion.

Our further observations indicated that the decrease in cellular proliferation is the result of ongoing differentiation. When the cells were cultured with TsA (500 nM) for 72 h, we noted a significant change in their shape – they became more spindle-like, resembling adult muscle cells (Fig. 4). What is more, the cells expressed genes
Fig. 1. WST-1 cell viability assay
IC50 was calculated for both cell lines. Cells were treated with rising doses of TsA for 72 h and stained with a stable tetrazolium salt. Results were normalized to the control non-treated cells. Panel A – RH30; Panel B – RD.

Fig. 2. Cell proliferation capacity assay conducted in two RMS cell lines, RH30 (Panel A) and RD (Panel B)
Increasing doses of TsA reduced the proliferation capacity of the cells to a near complete halt at 750 nM and 1 µM for RH30 and RD, respectively. Every 24 h medium was replaced with that containing a new dose of TsA. The results were calculated as % of control; control is the number of cells seeded at 0 h prior to TsA addition. *P < 0.05 indicates a significant decrease in proliferation potential as compared to unstimulated cells (0 nM – vehicle only).

Fig. 3. Representative study of apoptosis in TsA-treated RH30 and RD cells
Cells were treated with 0, 75, 300, 500 nM or 1 µM of TsA for 72 h and stained with propidium iodide and anti-annexin-V antibody. *P < 0.05 indicates a significant change in the number of live, early and dead cells as compared to unstimulated cells (0 nM – vehicle only).
characteristic of differentiated muscle at a much higher level than non-treated cells. By employing RQ-PCR and immunohistochemistry, we noted greater expression of CKM, MYL1, MYH1 and MYH2 (Figs. 4 and 5). Spindle-like cells were more abundant in the RH30 cell line vs. RD line. In control cultures without TsA, spindle-like cells were rare. After incubation with TsA, both cell lines showed increased amounts of myosin heavy chains (myosin hc) in comparison to control cells not treated with TsA. Stronger immunofluorescence was observed in the RH30 line in comparison to the RD line.

Sensitivity of the RMS cells to a differentiation-triggering agent such as TsA makes these cells more responsive to chemotherapeutic treatment. In Fig. 6, we show application of standard regimen chemotherapeutics (VAC) accompanied by 500 nM TsA for 72 h. Addition of TsA makes the cells more sensitive to rising doses of all three agents (actinomycin D, cyclophosphamide and vincristine). Specifically for vincristine, we see an additive effect as assessed by the fractional product method of Webb.

In order to better characterize the mechanism of TsA-induced differentiation, we performed screening microRNAs related to muscular differentiation – myomiRs. We noted that expression of most of already known myomiRs was reactivated upon TsA treatment as depicted in Fig. 7. In the group of selected microRNAs, we spotted miR-27b. In order to shed more light on the role of miR-27b, we transfected our cells with hsa-miR-27b-5p and hsa-miR-27b-3p (100 nM) for 48 h and noted that both RH30 and RD cells triggered the differentiation programme as visualized by rising expression of CKM, MYL1, MYH1 and MYH2 (Fig. 8).

![Fig. 4. RMS cells differentiate upon stimulation with TsA. The figure panels show immunodetection of myosin heavy chains (myosin hc) in RH30 (panel A and C) and RD (panels B and D) cell lines, in cultures with TsA (TsA+) and without TsA (Control – panels A and B). Scans captured with 40× objective computer zoom 2, scale bar 30 µm. Green fluorescence – myosin hc, blue fluorescence – nuclei stained with DAPI.](https://example.com)

![Fig. 5. RQ-PCR evaluation of RMS differentiation. The figure shows increased expression of genes characteristic of differentiated muscle cells. (CKM – creatine kinase muscle, MYL1 – myosin light chain 1, MYH1 and MYH2 – myosin heavy chain 1 and 2). *P < 0.05 indicates a significant increase in gene expression in TsA-stimulated cells (500 nM) as compared to unstimulated cells (ctrl).](https://example.com)
RMS derives from early skeletal muscle cells or myogenic precursors and displays characteristics of muscular differentiation. The cells express muscle-specific transcription factors such as MyoD and myogenin, but are not able to terminally differentiate (De Giovanni et al., 2009). They maintain the ability to proliferate indefinitely, giving rise to growing tumours and production of multiple metastases. Therefore, it is reasonable to work

Discussion

RMS derives from early skeletal muscle cells or myogenic precursors and displays characteristics of muscular differentiation. The cells express muscle-specific transcription factors such as MyoD and myogenin, but are not able to terminally differentiate (De Giovanni et al., 2009). They maintain the ability to proliferate indefinitely, giving rise to growing tumours and production of multiple metastases. Therefore, it is reasonable to work
Fig. 7. Upon TsA treatment, both cell lines re-expressed several miRNAs involved in muscular differentiation (myomiRs). RMS cells were treated with 500nM TsA for 48 h, and miRNA expression was evaluated by RT-qPCR. *Results are presented as fold differences and were calculated with the ΔΔCt method; expression in control, unstimulated cells equals 1. *P < 0.05 as compared with untreated control (ctrl).

**Fig. 8. Reactivation of RMS differentiation after transfection with miR-27b-5p and with miR-27b-3p**

The cells were transfected with 40 µM microRNA mimics for 48 h. Expression of genes characteristic of adult muscles was evaluated by RT-qPCR and calculated using the ΔΔCt method, whereas expression in cells transfected with negative control miRNA equals 1. Panel A – RH30; Panel B – RD. *P < 0.05 as compared to cells transfected with negative control miRNA.

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on strategies aimed at restoring the myogenic programme and reversing RMS cell malignant behaviour.

RMS is known for multiple epigenetic anomalies such as hypomethylation of the 11p15.5 chromosomal region or abnormal chromatin acetylation (Casola et al., 1997; Schneider et al., 2014). Previously, we have shown that application of epigenetic drugs such as azacytidine may be beneficial because they inhibit proliferation and alter expression of the genes involved in tumour growth and metastasis (Tarnowski et al., 2015). Namely, azacytidine-related demethylation causes down-regulation of growth-promoting IGF1 and reactivates non-coding H19 and related miR-675, which in turn targets the IGF1 receptor (IGF1R).

In the present study, we also focused on an epigenetic drug, but one with a different action. TsA is an old medicament, previously used as antifungal antibiotic, but it is also known for its strong effect on histone acetylation in eukaryotic cells. Treatment of wild-type myoblasts (of murine or human origin) with various HDAC inhibitors, such as TsA, valproic acid or sodium butyrate, all pan-inhibitors of class I and II HDACs, increases efficiency of myoblast fusion and favours myogenic differentiation (Sincennes et al., 2016).
A previous study conducted by Vleeshouwer-Neumann showed that two pan-HDAC inhibitors, TsA and suberoylanilide hydroxamic acid (SAHA; also known as vorinostat), are able to inhibit growth of RMS cancer cells, mostly by induction of differentiation (Vleeshouwer-Neumann et al., 2015). Similarly, in our study, we were able to see RMS cells differentiating into muscle tissue (Fig. 4) and over-expression of myogenic genes upon HDACi treatment. In the above-mentioned study, the authors focus their attention on ERMS cell lines as the effects on ARMS cells were little or variable. In our experiment, using different experimental settings (dosing, timing and cell density), we noted a negative effect of TsA on proliferation of both tested cell lines (RD and RH30).

However, in our case, we wanted to focus on different aspects of muscle differentiation and looked for any change in myomiR expression as a way of finding the mechanism that is responsible for this phenomenon. Our attention turned to miRNA and specifically to myomiRs as very potent inducers of muscle differentiation. Since their discovery, a cohort of miRNAs have been found to participate in the regulation of various cellular processes, including cell proliferation, differentiation and apoptosis (Wahida et al., 2010). In particular, spatial- and temporal-specific miRNAs serve as pivotal regulators of tissue determination, differentiation and maintenance (Honardoost et al., 2015).

We performed RT-qPCR profiling of the myomiRs previously known to be involved in RMS biology: miR-29, miR-302, miR-183, miR-133, miR-486, miR-206, miR-186, miR-450, miR-26, and miR-27b (Li et al., 2015) (Fig. 7). Most of the myomiRs were reactivated after TsA treatment.

Among the tested miRNAs, we also evaluated a novel myomiR – miR-27b. miR-27b has already been shown to affect Pax3 expression (Crist et al., 2009), inhibit skeletal muscle satellite cell proliferation but promote its differentiation, induce cardiac muscle cell hypertrophy (Wang et al., 2012), and regulate tumour cell proliferation, migration and invasion (Ding et al., 2017). The latest study by Hou et al. (2018) indicates that miR-27b promotes pig muscle satellite cell myogenesis by targeting MyoD family inhibitor (MDFI). In our work, we have shown that addition of exogeneous miR-27b triggers RMS cell differentiation, which was visualized by increased expression of genes characteristic of fully grown muscles such as CKM, MHC and MYL.

What is more, our study indicates that TSA, as well as decreasing on its own the growth of RMS cells, may also accompany the standard regimen of chemotherapeutics used for RMS treatment called VAC and comprising cyclophosphamide, actinomycin D and cyclophosphamide (Hosoi, 2016). For all of the drugs used here, TsA produced an additive effect, allowing reduction of the chemotherapeutics applied in the treatment. Similarly, it was shown that TsA can enhance sensitivity of cisplatin-resistant lung cancer cells to cisplatin (Wu et al., 2010). Another study found that in gastrointestinal malignant tumours, prostate, breast, lung cancer and ovarian cancers, TsA combined with cisplatin has a significantly higher inhibitory effect on cell proliferation than in single drug treatment groups (Zhang et al., 2006; Kanzaki et al., 2007; Chang et al., 2011; Zhang et al., 2015).

In summary, our study demonstrates that TsA or similarly acting HDAC inhibitors such as SAHA/vorinostat, depsipeptide or apicidin analogues may serve as very potent elements of combination therapy by inducing terminal differentiation of RMS cells and increasing sensitivity to conventional chemotherapeutics. It is important that HDAC inhibitors are characterized by few side effects towards normal cells; those side effects are reversible on drug cessation and primarily include fatigue, nausea, dehydration, diarrhoea, prolonged QT, thrombocytopaenia, lymphopaenia and neutropaenia (Rasheed et al., 2008). The mechanism through which they act involves reactivation of small miRNAs that may trigger a differentiation programme leading to the retardation of tumour growth.

References


