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SHORT REPORT

The antiretroviral nucleoside analogue Abacavir reduces cell growth and promotes differentiation of human medulloblastoma cells

Alessandra Rossi[1,2], Giuseppe Russo[1,3], Andrew Puca[1,2], Raffaele La Montagna[1,2], Mariella Caputo[1,4], Eliseo Mattioli[2], Massimo Lopez[5], Antonio Giordano[1,2,6] and Francesca Pentimalli[1,6]*

[1]*Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia PA*
[2]*Department of Human Pathology and Oncology, University of Siena, Siena, Italy*
[3]*DISI—Department of Computer and Information Sciences, University of Genoa, Genoa, Italy*
[4]*Department of Pharmacology, University of Salerno, Fisciano, Salerno, Italy*
[5]*Division of Medical Oncology B, Regina Elena Cancer Institute, Rome, Italy*
[6]*CROM—Center of Oncology Research Mercogliano, Avellino, Italy*

Abacavir is one of the most efficacious nucleoside analogues, with a well-characterized inhibitory activity on reverse transcriptase enzymes of retroviral origin, and has been clinically approved for the treatment of AIDS. Recently, Abacavir has been shown to inhibit also the human telomerase activity. Telomerase activity seems to be required in essentially all tumours for the immortalization of a subset of cells, including cancer stem cells. In fact, many cancer cells are dependent on telomerase for their continued replication and therefore telomerase is an attractive target for cancer therapy. Telomerase expression is upregulated in primary primitive neuroectodermal tumours and in the majority of medulloblastomas suggesting that its activation is associated with the development of these diseases. Therefore, we decided to test Abacavir activity on human medulloblastoma cell lines with high telomerase activity. We report that exposure to Abacavir induces a dose-dependent decrease in the proliferation rate of medulloblastoma cells. This is associated with a cell accumulation in the G_2/M phase of the cell cycle in the Daoy cell line, and with increased cell death in the D283-MED cell line, and is likely to be dependent on the inhibition of telomerase activity. Interestingly, both cell lines showed features of senescence after Abacavir treatment. Moreover, after Abacavir exposure we detected, by immunofluorescence staining, increased protein expression of the glial marker glial fibrillary acidic protein and the neuronal marker synaptophysin in both medulloblastoma cell lines. In conclusion, our results suggest that Abacavir reduces proliferation and induces differentiation of human medulloblastoma cells through the downregulation of telomerase activity. Thus, using Abacavir, alone or in combination with current therapies, might be an effective therapeutic strategy for the treatment of medulloblastoma.

Key words: abacavir; differentiation; medulloblastoma; reverse transcriptase; telomerase; senescence

At every round of cell division human chromosomes lose a certain amount of DNA at the telomeres, which is the basis of the limited replicative lifespan of normal cells.[1] Telomerase is a ribonucleoprotein enzyme consisting of 3 components, human telomerase RNA, telomerase-associated protein and human telomerase reverse transcriptase (hTERT). This enzyme utilizes its own RNA as template to synthetize telomeric DNA that, together with telomere-binding proteins, confers stability to chromosomes counteracting the telomere-dependent pathways of cell mortality.[1] Importantly, telomerase activity is detectable in over 80% of human tumour samples *in vivo*, including most of the common and therapeutically intractable types, and some studies have suggested that cancer stem or stem-like cells are also telomerase-positive.[2] Conversely, telomerase is not expressed in normal cells before birth and is expressed only transiently or at low levels in proliferative tissues after birth.[3] Thus, although telomerase itself is not considered an oncogene, its repression and tight regulation in humans function as tumour suppressor mechanisms, and it is now well-established that telomerase plays an important role in cellular immortalization and tumorigenesis.[4] The reactivation of telomerase in cancer cells stabilizes telomere length, thereby counteracting the cell division-related telomere erosion and providing unlimited proliferative capacity to malignant cells. This makes telomerase an attractive target for cancer therapy.[5] The key advantages of targeting telomerase in comparison with most other cancer targets are its wide expression in tumours and its specificity for cancer cells, including putative cancer stem cells.[1,6] No other tumour-associated gene is as widely expressed in cancer.[1,6] Furthermore, the low or transient expression of telomerase in normal tissues and the longer telomeres in normal cells, compared with those in tumours cells, reduce the probability of toxicity, associated with telomerase-based drugs, in normal cells suggesting that this kind of therapy could have a broad therapeutic window.[1] Moreover, as late stage and recurrent human tumours are characterized by immortal cells that have reactivated telomerase, telomerase antagonists may be useful also when traditional antitumour therapies, which are generally more effective against early stage cancer, have failed.[7] Thus, antitelomerase strategies promise to be a novel anticancer approach that might be effective also against disseminated advanced tumours.

Reverse transcriptase (RT) inhibitors belong to a family of 19 compounds used for the treatment of human immunodeficiency virus (HIV) infections.[8] They include the nucleoside RT inhibitors (NRTIs), the nucleotide RT inhibitors (NtRTIs) and the nonnucleoside RT inhibitors (NNRTIs).[8,9] After intracellular phosphorylation, NRTIs and NtRTIs function as competitive inhibitors of the normal substrates (either dATP, dGTP, dCTP or dTTP) and lead to the termination of chain elongation, thereby blocking DNA synthesis. Whereas NNRTIs inhibit RT activity by direct binding the hydrophobic pocket in the p66 subunit of RT enzyme, near the polymerase active site.[8,9]

Abacavir, (1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol succinate (ABC), also known as Ziagen, is a 2′-deoxyguanosine analogue, which has potent antiretroviral properties and therefore it has been approved for the clinical treatment of AIDS in adults and in children.[10] ABC is one of the most efficacious nucleoside analogues in the treatment of AIDS and it has a favorable safety profile and desirable pharmacokinetic characteristics, such as bioavailability.[11] In fact ABC is well and rapidly absorbed following oral administration and it has

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***Correspondence to:** CROM—Center of Oncology Research Mercogliano, "Fiorentino Lo Vuolo," Via Ammiraglio Bianco, 83013 Mercogliano, Avellino, Italy. Fax: +39-0825-1911706.
E-mail: francesca.pentimalli@gmail.com
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an absolute bioavailability of approximately 83%. ABC is metabolized, through a unique phosphorylation pathway, to a carbocyclic guanosine triphosphate, carbovir triphosphate (CBV-TP), which is a substrate for the HIV RT and causes DNA chain termination owing to the lack of 3′-OH.[12] According to Tendian *et al*.[13] the active form of ABC, which is a substrate for the HIV RT, is also an inhibitor of human telomerase activity. In fact CBV-TP is incorporated into DNA at the telomeres by telomerase, thereby blocking the extension of the DNA chain. This study was supported by the observation of Yegorov *et al*.[14] showing the inhibition of telomerase activity in mouse fibroblasts after exposure to CBV-TP, the active metabolite of ABC.

Telomerase expression is upregulated in 76% of primary primitive neuroectodermal tumours,[15] and in neuroblastoma the level of the enzyme correlates with high genetic instability and a poor clinical outcome.[1] More recently, Chang *et al*.[16] showed that telomerase activity is present in the majority of medulloblastomas, suggesting that telomerase activation is associated with the development of these diseases. Despite improvements in the overall survival rate of patients with medulloblastoma, owing to the multimodality treatment that includes surgery, radiation and chemotherapy, some patients will have recurrent or progressive disease.[17–19] Unfortunately, attempts to further reduce the morbidity and mortality associated with medulloblastoma are limited by the toxicity of conventional treatments and the infiltrative nature of the disease.[17] Thus, there is an urgent need to develop innovative therapeutic approaches that can improve survival and reduce toxicity. Therefore, we decided to test ABC activity on 2 human medulloblastoma cell lines, Daoy and D283-MED, in which a high telomerase activity is documented.[15,16] We report that exposure to ABC is associated with decreased cell growth rate in medulloblastoma cells and this effect is accompanied by the promotion of differentiation.

Material and methods

Cell cultures and Abacavir treatment

The human medulloblastoma cell lines Daoy and D283-MED were obtained from American Type Culture Collection (ATCC, Manassas, VA). Daoy cell line was established from a desmoplastic medulloblastoma from a 4-year-old male and grows as attached polygonal cells with a population doubling time of 30.5 hr (He *et al*.[20]). D283-MED was established from the peritoneal metastasis of a 6-year-old male with medulloblastoma and grows as a mixed culture of both attached and suspension cells.[21] These cell lines were routinely cultured in DMEM (CellGro, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biological, Norcross, GA) at 37°C in a humidified atmosphere of 5% CO_2 in air, according to the ATCC recommendations.

Abacavir was purified from commercially available Ziagen (GlaxoWellcome) by Dr. Raffaele La Montagna and dissolved in DMEM to make a 1 mg/ml stock solution. Cells were plated at the initial density of 700×10^3/100 mm diameter plate and 24 hr later were exposed to the drug. Cells were harvested by trypsinization at different time points and cell viability was assessed using the trypan blue dye exclusion method in a Burker chamber (3 counts per sample).

Cell cycle analysis

For cell cycle analysis, 1×10^6-aliquots of cells were harvested and washed twice with cold phosphate-buffered saline (PBS), fixed in 70% ethanol and stored at 4°C until the analysis. After centrifugation at 1,000 rpm, the resulting cell pellet was incubated, in the dark, in 0.3 ml of freshly prepared PBS containing 0.02 mg/ml propidium iodide and 0.25 mg/ml ribonuclease A (Sigma, Sigma-Aldrich). The DNA content of the cells was analyzed using a FACStar Plus flow-cytometer (Beckton-Dickinson) (10,000 events/sample).

Telomerase activity assay

Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP).[22] To this purpose, cells were treated with 750 μM of Abacavir for 1, 2, 3, 4 or 5 days and were washed once in PBS. Then the pellets ($\sim 1 \times 10^5$ cells) were suspended in 400 μl of TRAPeze CHAPS lysis buffer (Chemicon International, Temecula, CA). The samples were incubated for 30 min on ice and then centrifuged for 30 min (14,000 rpm, 4°C). The supernatant was collected, snap-frozen and stored at −80°C. Protein concentration of the extract was measured using the Quick Start Bradford Dye Reagent (Bio-Rad Laboratories, Hercules, CA). For each sample 4 μg of proteins were incubated with 75 mM Tris-HCl (pH8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 200 μM dNTPs, 100 ng of TS primer (5′-AATCCGTCGAGCAGAGTT-3′) in a thermocycler for 31 min at 30°C for the generation of telomeric repeats. After heating at 94°C for 5 min and cooling at 72°C, 2.5 U of Taq DNA polymerase (Fermentas, Life Sciences), 100 ng ACX return primer (5′-GCGCGG $(CTTACC)_3$CTAACC-3′), 100 ng of NT internal control primer (5′-ATCGCTTCTCGGCCTTTT-3′), and 0.02 amol of TSNT internal control (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′) were added to a total reaction volume of 50 μl. Thirty PCR cycles (94°C for 40 s, 56°C for 55 s and 72°C for 55 s) were performed and the PCR products were electrophoresed on a 10% polyacrylamide nondenaturing gel in TBE. Electrophoresis was carried out at 200V in TBE at room temperature until the bromophenol blue just ran off the gel. Then, the gel was analyzed following silver staining according to Dalla Torre *et al*.[23] The appearance of a 6-nucleotide ladder starting at 50bp indicates the presence of telomerase activity. The TRAP assay included the amplification of an internal telomerase assay standard band (ITAS), a primer-dimer/PCR control and a negative control from Daoy and D283-MED cell lines after heat inactivation (85°C for 10 min).

Immunofluorescence detection of glial and neuronal differentiation markers

After 96 hr of treatment with ABC, Daoy and D283-MED cells were fixed in 3.7% (v/v) formaldehyde/PBS and permeabilized with 90% methanol/PBS (v/v). Then, cells were incubated with 1 μg/ml of monoclonal antibody against the glial fibrillary acidic protein (GFAP) or polyclonal antibody against the neuronal marker synaptophysin (SYN) (Santa Cruz Biotechnology, Santa Cruz, CA), washed and incubated with secondary FITC-conjugated antibodies (1/1,000, Vector Laboratories, Burlingame, CA). The samples were mounted in Vectashield (Vector laboratories) containing 4,6-Diamidino-2-Phenylindole (DAPI) in order to visualize the nuclei, then analyzed by fluorescence microscopy.

Real Time RT-PCR

To analyze the expression levels of *GFAP*, *SYN* and *hTERT* mRNAs during the treatment of ABC, real-time RT-PCR was performed with Power SYBR green PCR master mix (Applied Biosystems, Foster city, CA) in Real-Time PCR Systems 7500 Fast (Applied Biosystems, Foster city, CA). Daoy and D283-MED cells were harvested after 4, 8, 12, 24, 48, 72 and 96 hr of treatment with Abacavir (750 μM) and total RNAs were extracted with Nucleospin RNA II (Macherey-Nagel) in diethyl pyrocarbonate-treated water. cDNA was synthetized with M-MuLV RT RNase H^- (Finnzymes, Finland) and 100 ng random hexamers in a total volume of 20 μl from 1 μg of total RNA. For PCR the following primers were used: for *GFAP* (accession No.:NM_002055) forward 5′-TGGAAGCCGAGAACAACCT-3′, reverse 5′-CCTCCAGCGACTCAATCTTC-3′; for *SYN* (accession No.:NM_003179.1) forward 5′-GTGACCTCGGGACTCAACAC-3′, reverse 5′-AGCCTGTCTCCTTAAACACGAA-3′ and for *hTERT* (accession No.:NM_198253) forward 5′-CGGAAGAGTGTCTGGAGCAA-3′, reverse 5′-GGATGAAGCGGAGTCTGGA-3′. Gene expression level was normalized to the value of the internal standard *HPRT1* (accession No.:NM_000194) forward 5′-AGCCAGACTTTGTTGGATTTG-3′, reverse 5′-TTTACTGGCGATGTCAATAAG-3′, and the differences in gene expression were calculated by the standard ΔΔCt method. All the reactions were performed twice in triplicate and the specificity was assessed by anal-

ysis of the dissociation curve generated from each reaction and electrophoretic run on a 2% agarose gel.

Senescence-associated β-galactosidase activity

SA-β-Gal activity was determined using β-galactosidase staining. Cells were washed in PBS, fixed for 3 to 5 min (room temperature) in 3% formaldehyde, washed, and incubated at 37°C (in absence of CO_2) with fresh SA-β-Gal stain solution: 1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (Promega), 40 mM citric acid, sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$. Staining was evident in 12 to 16 hr.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of three independent experiments and statistical significance was evaluated using Student's *t* test. For each statistical analysis, the associated probability (*p* value) of <5% was considered significant. Statistical differences between controls and samples were indicated by * for $p < 0.05$ or ** for $p < 0.01$.

Results

Abacavir inhibits the growth of medulloblastoma cells in vitro

As a first step to test the effect of ABC in the human medulloblastoma cell lines Daoy and D283-MED, we analyzed the growth rate of cells exposed to different doses of the drug compared with nonexposed controls. We decided to start with the doses used for the non nucleosidic RT inhibitor nevirapine, which has demonstrated cytotoxicity *in vitro* in melanoma and prostate cancer.[24] As shown in Figure 1*a*, a dose-dependent effect of ABC on the cell growth rate of cells was observed, with the maximal growth suppression at the 550 to 750 μM dose range, an intermediate response at 350 μM, and no suppressive effect below the 250 μM dose (data not shown), in the cell lines tested.

At the doses of 550 and 750 μM of ABC we observed a massive decrease in cell density at early time points, which was accompanied by an evident change in morphology. Cells appeared much larger in size and grew as monolayer with evidence of neurite formations (Fig. 1*b*).

Next, we analyzed by flow cytometry the cell cycle of cells treated with the different doses of ABC, to test whether the exposure to the drug has also effects on the cell cycle. Figure 2 shows the cell distribution across the phases of cell cycle at different time points during ABC treatment. At 48 hr of exposure to ABC, Daoy cells accumulated in the S and G_2/M phases of cell cycle, in a dose-dependent manner. At the maximal dose of the drug we observed: 32.3% in S phase, 37.3% in G_2/M phase for ABC-treated cells *vs*. 21.5% and 21.8%, respectively, for untreated cells. Interestingly, at 72 hr interval, we observed a complete shift of treated cells into G_2/M phase: 26.9% in S phase, 37.8% in G_2/M phase *vs*. 16.8% in S phase and 19.0% in G_2/M phase in control cells and, after 2 weeks of treatment, ABC induced cell death (Fig. 2*b*). The cytotoxic effect was more pronounced in D283-MED. In fact, as evidenced by FACS analysis, cells with a sub G1 DNA content accumulated after 48 hr of treatment with all doses of ABC. A maximal effect was observed with 750 μM dose of ABC in both cell lines (Fig. 2*b*). Therefore, we focused our studies on the 750 μM dose of ABC. To test the effect of ABC and its eventual cytotoxicity on normal cells, we used primary cultures of rat neurons. We did not observe morphological changes such as loss of neuronal processes, at the doses of 550 and 750 μM, as evidenced by the photographs in Figure 1*c* obtained before and after exposure to 750 μM ABC. Given that neurons are non proliferative cells, we have also tested whether ABC has effects in the cell cycle of the immortalized neuronal cell line, HT22, from mouse hippocampus. No effect was observed in the cell cycle profile of ABC-treated cells compared with untreated cells, also at the higher dose tested (Fig. 2*c*).

Abacavir induces the downregulation of telomerase activity in medulloblastoma cells

To assess the effect of ABC on telomerase activity, we performed a TRAP assay in both cell lines at 1, 2, 3, 4 and 5 days after treatment with 750 μM of ABC. We found that the antiproliferative effect of ABC is accompanied by the inhibition of telomerase activity as evaluated by the decrease of the 6bp ladder beginning from 1 day of treatment (Fig. 3*a*). Interestingly, also the level of *hTERT* mRNA, encoding the catalytic subunit of telomerase, was downregulated as early as 1 day after ABC treatment, as shown by real-Time RT-PCR (Fig. 3*b*). This finding suggests that ABC might inhibit telomerase activity not only by acting as guanosine analogue in DNA synthesis at the telomeres, as previously reported,[13] but also through the downregulation of *hTERT* mRNA transcription.

Exposure to Abacavir promotes differentiation in medulloblastoma cells

According to Gao *et al*.[25] telomerase is repressed during lineage stem cell maturation in embryonic development and during differentiation of various immortal cells,[26–28] suggesting that differentiation is associated to the downregulation of telomerase activity. Therefore, we investigated whether ABC is able to influence also the process of differentiation in Daoy and D283-MED cells. Medulloblastoma cells express various markers of cell differentiation, such as those of the neuronal and glial lineage, but also of cartilage, muscle, fat and mesenchyme.[29,30] Furthermore, it was shown that adjuvant therapy can induce extensive or complete neuronal maturation in medulloblastoma in 2 medulloblastoma cases, suggesting that these tumour cells can differentiate.[31]

After ABC exposure, we detected in both cell lines analyzed, by immunofluorescence staining, increased protein expression of SYN and GFAP, which is one of the intermediate filaments found in mature normal astrocytes and differentiated glioma cells. SYN and GFAP increase became apparent in cells after 3 days of exposure to 550 and 750 μM of ABC, as indicated in Figure 4*a*. Whereas, at lower doses (350 μM), the drug causes only a marginal increase in GFAP and SYN protein levels after 1 week (data not shown). As expected, no or low levels of protein expression were found, instead, in untreated cells. Real-Time RT-PCR confirmed the pattern of increased gene expression of both *GFAP* and *SYN* in Daoy cells after 1 day of exposure and in D283-MED at earlier time of treatment (Fig. 4*b*). These data are consistent with the morphologic changes and the neurite formation described above and showed in Figure 1*b*.

Abacavir induces senescence in medulloblastoma cell lines

Telomerase inhibition gives rise in cells to a telomere-induced senescence that is triggered by the formation of critically short and dysfunctional telomeres.[27] Cellular senescence is a tumour suppressive mechanism that mediates the anticancer effects of many chemotherapies.[32] Interestingly, one of the morphologic changes that was observed in our ABC-treated cells is the flattening of cells, which is a typical morphologic change associated with cellular senescence.[32] By β-galactosidase staining, we confirmed that cellular senescence was induced in D283-MED and Daoy cells by ABC in a time- and dose-dependent manner beginning from 1 week of treatment. Both cell lines showed a substantial senescent phenotype also at the dose of 350 μM as shown in Figure 4*c*.

Discussion

Telomerase is a relatively specific cancer target as normal body cells express little or no telomerase for most of their lifespan and generally have longer telomeres compared with tumour cells.[1]

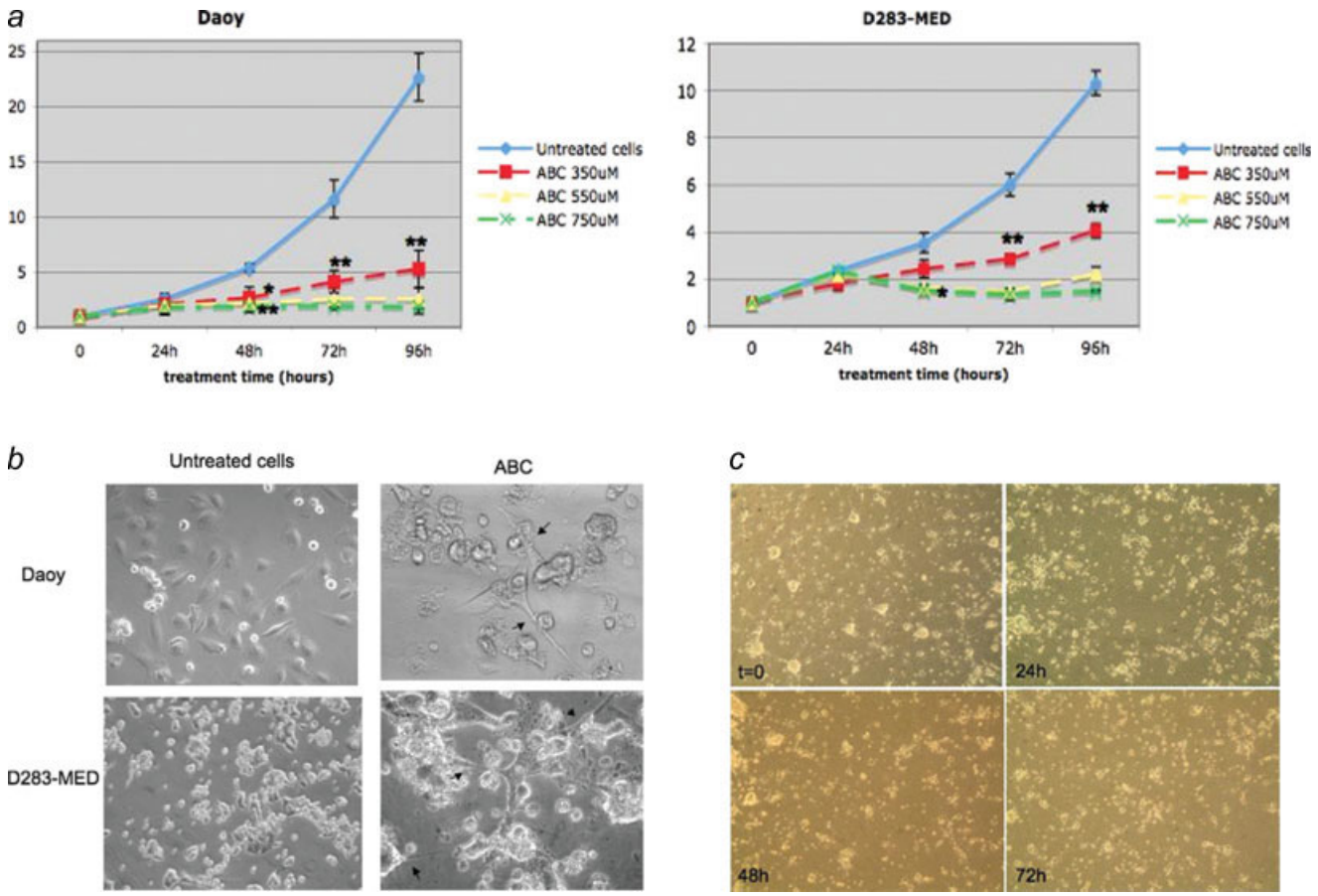


Figure 1 – Proliferation of human medulloblastoma cell lines Daoy and D283-MED treated with Abacavir (ABC). (*a*) Cells were cultured with different doses of ABC (dashed lines) and in drug-free medium (solid line). The cell proliferation rate is indicated as the ratio of cell number at each time point relative to the initial number of seeded cells, taken as 1. These results are expressed as the mean value of at least 2 independent assays; *bars*, ± SD. The (*) and (**) symbols denote significance difference of $p < 0.05$ and $p < 0.001$, respectively, from untreated cells control. (*b*) ABC induces morphologic differentiation in medulloblastoma cells. Daoy and D283-MED cells were cultured in the presence of 750 μM ABC for 2 weeks; ABC-containing fresh medium was changed at 72 hr intervals. *Arrows*, neurite formations. Photographs were obtained by phase contrast microscopy. (*c*) Morphologic features of neurons in primary culture on phase-contrast microscopy. Cells were treated with ABC (750 μM) for 24, 48 and 72 hr. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ABC is a specific RT inhibitor that has been approved for clinical use in the treatment of AIDS and is reported to be well tolerated in patients.[33] Furthermore, it has been shown that ABC exerts an inhibitory effect on the telomerase activity. In this report, we showed that ABC inhibits cell growth of the human medulloblastoma cell lines Daoy and D283-MED and this effect is associated with the downregulation of telomerase activity. To date, 2 major therapeutic approaches against telomerase-positive tumour cells are in clinical trials. GRN163L, a direct telomerase inhibitor, is in trial for the treatment of chronic lymphocytic leukaemia, multiple myeloma, solid tumours and nonsmall cell lung cancer (NSCLC).[34] Several therapeutic vaccines targeting hTERT are in, or have completed, trials for the treatment of leukaemia and renal, prostate, lung, skin, pancreatic and breast cancer.[1] Compared with other tested compounds targeting the telomerase, or telomerase therapeutic vaccines, which are currently in clinical trials, ABC has been used for many years for the treatment of AIDS.[10,35] Therefore, the prospect of using this drug in cancer therapy would have obvious advantages given its favourable safety profile and its epidemiological record of generally good tolerance to continued administration. Indeed, we did not observe cellular toxicity, after ABC treatment, in 2 normal neuronal cell types of murine origin.

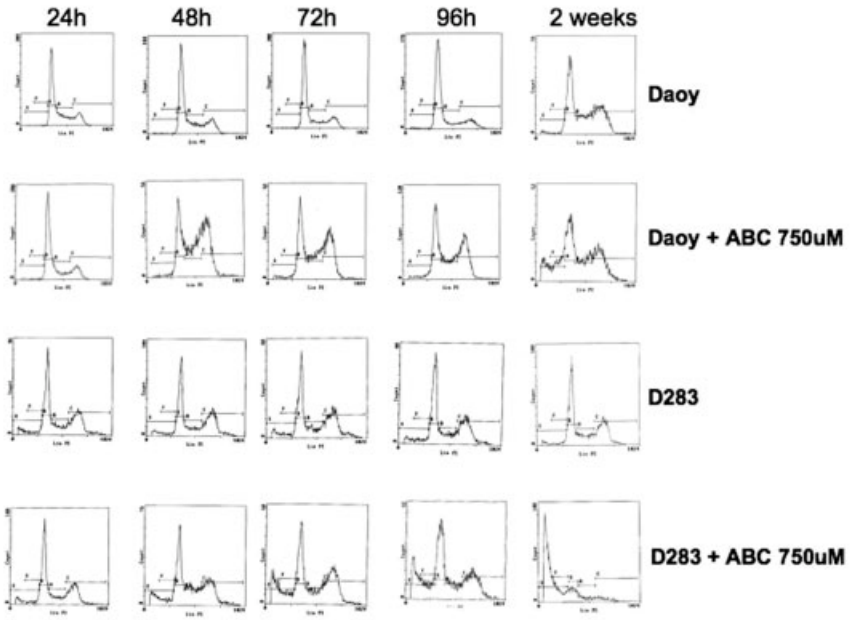
Here, we observed that the ABC-dependent inhibition of cell proliferation in D283-MED cells is accompanied by a dramatic increase in cell death. This is in accordance to another study showing that morphogenetic protein-7 (BMP7) induces telomerase inhibition, telomere shortening, and cell death by a mechanism involving the repression of hTERT, in cervical cancer cells.[36]

Whereas, in the Daoy cell line, along with the decrease in cell proliferation, we observed a significant cell accumulation in the G_2/M phase of the cell cycle, after exposure to ABC. ABC did neither cause significant cell death nor apoptosis as indicated by FACS measurement of cells with hypodiploid DNA content, suggesting that decreased cell proliferation rather reflects a progressive withdrawal from the cell cycle. Telomerase is a ribonucleoprotein complex that adds telomeric repeats onto the ends of chromosomes during the replicative phase of the cell cycle. In fact, the highest levels of telomerase activity are found in cells that are in the S-phase of the cell cycle, whereas cells in G_2/M phase have little or no telomerase activity.[37] This is in accordance to other studies showing that the inhibition of telomerase activity is associated with a block in the G_2/M phase of the cell cycle.[38] One of these studies showed that phorbol 12-myristate 13-acetate induced a cell cycle arrest in G_2/M in asynchronously growing NSCLC cells and conferred morphological and biochemical features of senescence, including downregulation of telomerase activity.[39] Our results show that ABC treatment leads Daoy cells to arrest in G_2/M whereas it induces cell death in D283-MED, likely through the inhibition of the telomerase. However, further studies to examine the molecules implicated in the regulation of the G_2/M phase

a

		Daoy				D283			
		G0	G2/M	S	Cell death	G0	G2/M	S	Cell death
24h	Untreated cells	55,9	19,2	24,4	0,73	41,4	34,0	15,7	8,72
	ABC 350uM	52,1	21,2	25,6	1,16	31,0	29,4	25,2	14,4
	ABC 550uM	55,4	20,1	23,1	1,56	35,2	31,4	23,3	10,0
	ABC 750uM	60,5	19,5	18,3	1,84	45,4	30,0	14,9	9,61
48h	Untreated cells	55,1	21,8	21,5	1,62	39,6	37,8	14,7	7,78
	ABC 350uM	45,4	27,2	24,9	2,53	38,5	29,4	17,5	14,6
	ABC 550uM	35,1	33,4	29,3	2,38	30,0	36,3	19,1	14,5
	ABC 750uM	35	37,3	32,3	1,84	32,2	30,3	23,1	14,4
72h	Untreated cells	62,8	19	16,8	1,54	37,7	37,1	14,6	10,2
	ABC 350uM	44,6	24,7	26,1	4,92	33,8	28,0	16,0	21,9
	ABC 550uM	40,1	31,8	24,3	3,95	29,2	22,8	15,6	32,4
	ABC 750uM	30,3	37,8	26,9	4,92	29,3	33,4	15,2	22,1

b



c

		HT22			
		G0	G2/M	S	Cell death
24h	Untreated cells	45,7	16,4	25,6	11,4
	ABC 550uM	37,3	18,2	31,2	12,8
	ABC 750uM	36,6	19,6	29,4	14,0
48h	Untreated cells	45,5	20,9	21,5	11,4
	ABC 550uM	42,1	16,1	26,4	14,5
	ABC 750uM	38,5	17,0	26,4	17,4
72h	Untreated cells	45,1	22,3	25,2	6,91
	ABC 550uM	42,1	22,8	21,0	13,5
	ABC 750uM	40,3	22,8	23,1	13,4

Figure 2 – Flow cytometric profiles of Daoy and D283-MED medulloblastoma cells and HT22 neuronal cell line before and following exposure to Abacavir (ABC). (*a*) The percentages of cells in the different phases of cell cycle are reported for each representative time point. The data shown are representative of at least 2 independent experiments. (*b*) FACS analysis was performed after 24, 48, 72, 96 hr and 2 weeks following 750 μM ABC treatment. A consistent shift of the cell population toward the G_2/M phase of the cell cycle was observed in treated Daoy cells and an increased percentage of cell death was evidenced in D283-MED. (*c*) No differences in cell cycle profile were evidenced in HT22 cells treated with 550 or 750 μM ABC, compared to untreated cell.

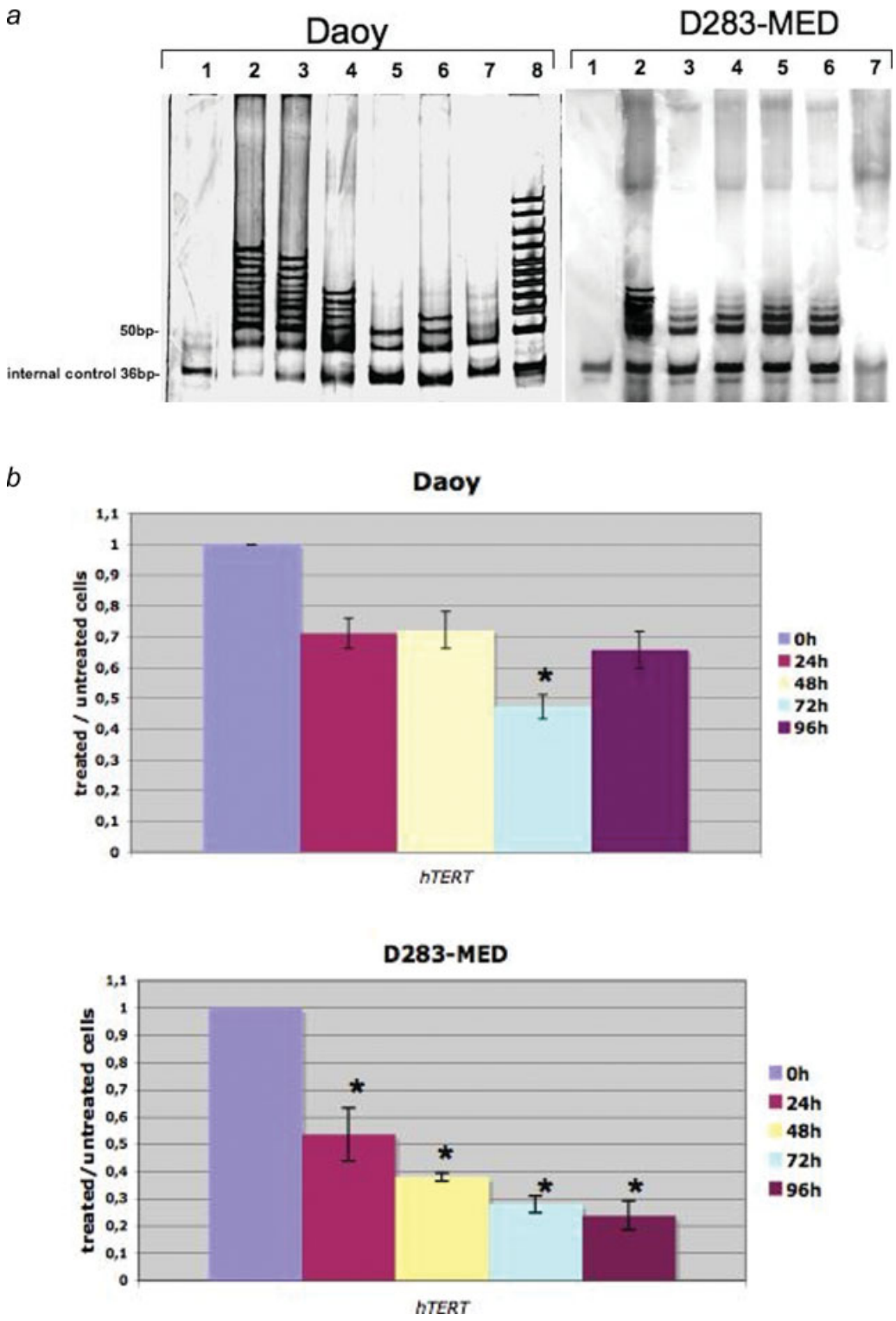


FIGURE 3 – Abacavir (ABC) inhibits telomerase activity and downregulates *hTERT* mRNA. (*a*) Detection of telomerase activity in medulloblastoma cells by TRAP assay. The detection of 6bp ladders indicates the presence of telomerase activity. Daoy and D283-MED cells are telomerase positive and heat treatment of the protein extract abolished the telomerase activity, as assessed by the TRAP assay. A reduction of telomerase activity was observed in both cell lines beginning from 1 day of treatment with 750 μM of ABC. Lane 1, heat-treated protein extracts from Daoy or D283-MED; lane 2, untreated cells; lane 3, 4, 5 and 6, cells treated with ABC for 24, 48, 72 and 96 hr; lane 7, the primer-dimers/PCR control; lane 8, 100bp DNA ladder. (*b*) Expression of *hTERT* mRNA in Daoy and D283-MED cells was determined by real time RT-PCR. Data shown are representative of at least 2 independent experiments; *bars*, ±SD. The (*) symbol denotes significance difference of $p < 0.05$ from untreated cells control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

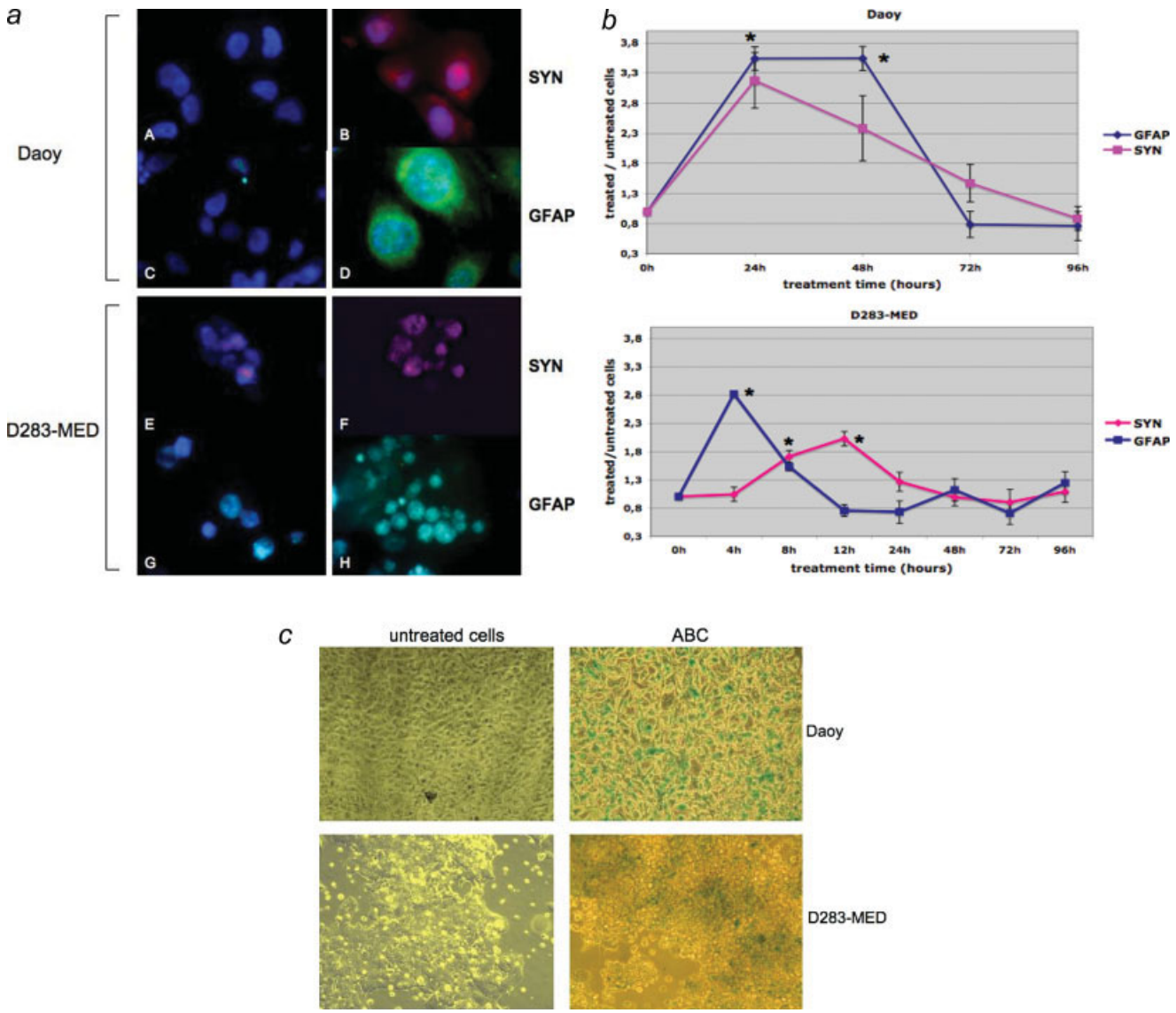


Figure 4 – Abacavir (ABC) induces differentiation and cellular senescence in medulloblastoma cells. (*a*) Immunofluorescence staining for GFAP and SYN proteins in Daoy and D283-MED cells. Expression of GFAP and SYN protein was evident in cells treated with 750 μM ABC for 3 days (B,D,F,H) respect to untreated cells, which are negative for or have lower levels of these proteins (A,C,E,G). All photomicrographs were taken with the same magnification. (*b*) *GFAP* and *SYN* mRNA levels as detected by real-time RT-PCR in medulloblastoma cells during the treatment with ABC. Both *GFAP* and *SYN* mRNA expression increased after exposure to the drug. Data shown are representative of at least 2 independent experiments; *bars*, ±SD. The (*) symbol denotes significance difference of $p < 0.05$ from untreated cells control. (*c*) β-galactosidase staining in Daoy and D283-MED after 1 week of treatment with 350 μM ABC, and the corresponding controls (untreated cells). Blue staining indicates senescent cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

checkpoint and in the cell death programme are crucial to understand the molecular mechanism of ABC, and they are currently under investigation in our laboratory.

Consistent with the central role of telomere shortening in the replicative senescence programme,[32,39,40] ABC was also able to induce cellular senescence in both medulloblastoma cell lines. This is very interesting considering that senescence is a powerful tumour suppressive mechanism. But whether the senescent phenotype is a direct consequence of telomere erosion, or rather it is due to telomere length-independent changes remains to be established. In fact, it has been reported that after telomerase repression other mechanisms that are independent from telomere erosion can trigger a damage response and abrupt cell-cycle arrest or death.[1,41,42]

Moreover, the elevated expression of GFAP and SYN markers, both at mRNA and protein levels, along with the neurite formation observed in both cell lines following 2 weeks of treatment with ABC, suggested that a more differentiated phenotype had been induced. The induction of differentiation occurring in our experiments after ABC treatment is typical of others inhibitors of telomerase activity and is likely to be due to an altered pattern of gene expression.[43] According to Damm *et al.*,[43] in fact, the drug-mediated telomerase inhibition in cancer cells has been reported to reduce proliferation and induce changes in gene expression, along with the appearance of senescence hallmarks. Our results are in accordance to another study, in which anticancer effects of aloe-emodin involve G_2/M cell accumulation concomitant with the induction of differentiation.[44] In addition, retinoic acid (RA) derivatives showed induction of cell differentiation, apoptosis, and growth arrest in some medulloblastoma cells. In particular, the results demonstrated that cell growth arrest was the main mecha-

nism by which RA inhibited cell proliferation in the medulloblastoma cell line Daoy, but not in the others (D283-MED and D341), in which it induces apoptosis.[45] Therefore it is possible that ABC inhibits cell proliferation, by G2/M block or cell death depending on cell type, and induce differentiation through the inhibition of telomerase activity. Even if the molecular mechanism by which ABC inhibits proliferation and induces differentiation are still to be clarified, the finding that ABC can induce the expression of differentiation markers in medulloblastoma cells suggests that it may attenuate the malignant and aggressive phenotype of transformed cells slowing the progression of disease. Molecular oncology studies, in fact, have shown that the genes regulating the differentiation process are not lost in malignant cells but the signals regulating cell proliferation, apoptosis and differentiation are defective. Thus, it is important to find agents that are able to restore the regulating machinery that will induce tumour cells to differentiate and revert their malignant phenotype. Interestingly, growth inhibition and increased sensitivity to anticancer drugs were observed in Daoy cells transfected with *GFAP*.[46] Such differentiation could also make tumour cells more responsive to normal growth regulatory signals or more sensitive to chemotherapy and other differentiation agents.[47] For instance, the remarkable success of all-*trans*-retinoic acid in producing complete remission in patients with acute promyelocytic leukemia has attracted interest in differentiation as an alternative form of cancer chemotherapy.[48] Treatment with retinoids can also induce differentiation in astrocytes, together with telomerase activity downregulation and increase of cell sensitivity to taxol.[49]

Another hurdle in the treatment of medulloblastoma is that the blood-brain barrier restricts the entry of hydrophilic and large lipophilic compounds into the brain.[17,35] The lipophilic nature of ABC, which enables it to pass through the blood-brain barrier more easily,[50] represents yet another advantage of its possible use for the treatment of medulloblastoma. Moreover, given its good tolerability, ABC treatment could be associated to current therapies used for medulloblastoma, including chemotherapeutic alkylating agent, such as cisplatinum and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, or mitotic inhibitors, such as vincristine.[17,51]

In conclusion, we suggest that the use of ABC, which is currently used in the treatment of AIDS, could be an effective therapeutic strategy, alone or in combination with current therapies, for the treatment of telomerase expressing tumours, such as human medulloblastomas.

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