Histone Deacetylase Inhibitors, but not Vincristine, Cooperate with Radiotherapy to Induce Cell Death in Medulloblastoma

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Abstract. Background: Though ionising radiation (IR) is an efficient means of postoperative treatment for children with medulloblastoma, the disease is incurable in about a third of them. Thus, multimodality regimens have been introduced, typically combining IR with vincristine. Materials and Methods: The combination of IR and vincristine was compared to the combination of IR and histone deacetylase inhibitors (HDIs) for their anticancer activity against medulloblastoma cells in vitro. Cytotoxic activities were assessed by measuring propidium iodide uptake and by cell cycle analysis. Results: HDIs augmented the cytotoxic effect of IR, while the combination of vincristine and IR was significantly less cytotoxic than vincristine alone. Cell cycle analyses revealed that vincristine did not interfere with IR-induced G2/M arrest, whereas HDIs abolished the latter. Conclusion: These in vitro findings indicate a favourable interaction of IR and HDIs, but an unfavourable one of IR and vincristine, in medulloblastoma, and provide a rationale for comparing the combination of IR with either vincristine or HDIs in vivo.

Medulloblastoma is a frequent brain tumour of childhood. It is a highly malignant disease that leads to death in more than a third of patients within five years of diagnosis (1). The therapy for medulloblastoma consists of a multimodality treatment with surgery, ionising radiation (IR), and chemotherapy. In particular IR is a very efficient therapy, however, it is associated with severe neurological toxic side-effects (2). In order to reduce morbidity with IR, in the USA, the standard craniospinal radiation dose has been lowered from 36 Gy to 23.4 Gy by the introduction of adjuvant chemotherapy (1). Nevertheless, a dose of 23.4 Gy is still associated with neurocognitive sequelae, particularly in children younger than seven years (3, 4). A more effective treatment regimen, aiming at decreasing IR-related morbidity without reducing disease control, is thus highly desirable.

Cells in the G2/M phase of the cell cycle are especially sensitive to IR (5). Therefore, an arrest of cells in the G2/M phase with antimitotic drugs, such as vinca alkaloids like vincristine, may improve the efficacy of IR. Preclinical studies of the combination of IR and vinca alkaloids for the treatment of different carcinomas have, however, produced contradictory results (6-11). Notably, in a study of tumour bearing mice, vinblastine has been shown to protect cells against IR-induced DNA strand breaks (9). In a study of carcinoma cell lines, IR antagonised vincristine- and vinblastine-induced apoptosis (11). Hence, a favourable interaction of IR and vinca alkaloids is not yet clearly established.

Histone deacetylase inhibitors (HDIs) are another class of anticancer agents that hold promise for enhancing the effectiveness of IR. HDIs function by inhibiting histone deacetylases, resulting in the accumulation of acetylated histones, in turn leading to relaxation of chromatin concomitant with an increase in transcriptionally active genes (12). As relaxed chromatin has been shown to be more sensitive to IR (13, 14), HDIs may improve the response to IR. Furthermore, HDIs have been reported to inhibit DNA repair activity, thereby increasing the sensitivity of cancer cells to IR (15, 16).

In a previous report, we have shown that HDIs potently enhanced the efficacy of IR in a medulloblastoma cell line, DAOY (17). Here, these studies have been extended to another medulloblastoma cell line, UW228-2. For comparison, the combination of vincristine and IR was also evaluated for its effectiveness in both medulloblastoma cell lines.
Materials and Methods

Reagents. Suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) were purchased from Alexis (Grüneberg, Germany). Vincristine was purchased from Sigma (Deisenhofen, Germany). SAHA and TSA have previously been shown to induce hyperacetylation of histone H3 in DAOY and UW228-2 cells (17).

Cell culture. DAOY and UW228-2 medulloblastoma cells were a gift from Dr. M. Grotzer (Zurich, Switzerland) and were maintained in Improved MEM Zinc Option (Invitrogen, Karlsruhe, Germany) or Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulphate (DMEM and supplements were purchased from Biochrom, Berlin, Germany). Cells were cultivated at 37°C in a humidified 5% CO2 incubator and routinely passaged when 90-95% confluent. Cell viability was determined by the trypan blue exclusion test. Cells were regularly inspected to determine freedom from mycoplasma with mycoplasma detection reagents from Roche (Mannheim, Germany).

Ionising radiation. Cells were exposed to IR four hours after treatment with SAHA or TSA or with vincristine using a Siemens Mevatron MXE linear accelerator (Siemens, Concord, CA, USA) delivering photon beams of 6 MV.

Cytofluorometric analysis of cell death. To determine cell death, cells were harvested after a 24-h (DAOY) or a 48-h (UW228-2) cultivation after exposure to IR, followed by a 5-min incubation in 2 µg/ml propidium iodide (PI) (Sigma) in phosphate buffered saline (PBS) at 4°C in the dark. PI uptake was assessed by flow cytometry analysis on a Becton Dickinson (Heidelberg, Germany) FACSCalibur using CellQuest software. 10,000 cells were analysed in each sample; data were gated to exclude debris.

Cytofluorometric analysis of DNA content. To measure DNA content, cells were analysed for PI incorporation into DNA. Cells were harvested 24 h (DAOY) or 48 h (UW228-2) after exposure to IR, washed twice with PBS and fixed in 70% ethanol at −20°C for at least 30 min. After centrifugation, cells were resuspended in PBS containing 1% glucose, 50 µg/ml RNase A (Roche) and 50 µg/ml PI and incubated in the dark at room temperature for 30 min. Flow cytometry analysis was performed on a FACSCalibur using CellQuest software. 20,000 cells were analysed in each sample; data were gated to exclude debris. Sub-G1, G1 and G2/M phase cells were calculated from the DNA content histograms.

Statistical analysis. Statistical significance of differences between experimental groups was determined using the paired two-tailed Student’s t-test.

Results

HDIs sensitize UW228-2 cells to ionising radiation. As shown in Figure 1A, the doses of HDIs (4 µM SAHA or 0.2 µM TSA) applied induced cell death in approximately 30% of cells. Treatment with IR alone was of little effect: doses of 20 or 40 Gy elicited 13% and 20% cell death, respectively.

Figure 1. HDIs interact cooperatively with IR. UW228-2 cells were irradiated 4 h after treatment with HDIs and cultured for a further 48 h. (A) Cytofluorometric analysis of PI uptake. (B) Cytofluorometric cell cycle analysis. Means±SD of 3 experiments are shown (**p<0.005).
However, combined treatment with HDIs and IR resulted in supra-additive cytotoxicity with 41-57% cell death at 20 Gy and 63-66% cell death at 40 Gy. This potentiation of cell death of the combined treatment with HDIs and IR was further confirmed using cell cycle analysis to assess DNA fragmentation; the sub-G₁ (hypodiploid) subpopulation of cells is indicative of apoptosis. As illustrated in Figure 1B, treatment with IR alone caused only a weak increase in sub-G₁ cells (16% at 20 Gy and 12% at 40 Gy). In contrast, in cells pretreated with SAHA or TSA for 4 h, IR of 20 or 40 Gy provoked DNA fragmentation in 27-32% and 52%, respectively, of the cells. An additional finding of the cell cycle analyses was that IR alone caused a strong elevation of the G₂/M peak: in the untreated control, 29% of cells were in the G₂/M phase of the cell cycle while 50% or 70% of cells were found in the G₂/M phase after exposure to 20 or 40 Gy, respectively (p<0.005). Interestingly, this G₂/M arrest was largely abrogated when cells were pretreated with HDIs.

Ionising radiation antagonises vincristine-induced cell death in UW228-2 and DAOY cells. As presented in Figure 2A, non-pretreated cells showed some responsiveness to IR, with maximally 21% or 28% cell death in UW228-2 and DAOY cells, respectively. Vincristine alone elicited cell death in up to 36% of UW228-2 cells and up to 33% of DAOY cells. However, the cytotoxic effect of vincristine was markedly impaired when it was combined with IR. Cell death induced by 30 nM vincristine was significantly (p<0.005) reduced to maximally 19% by exposure to 20-40 Gy in UW228-2 cells and 5-20 Gy in DAOY cells compared to 30 nM vincristine without IR. In fact, at doses of 20-40 Gy, vincristine did not add at all to the overall cytotoxicity of IR. Second, cells were assessed for DNA fragmentation by cell cycle analysis. Treatment with IR alone resulted in DNA fragmentation in maximally 17% or 34% in UW228-2 and DAOY cells, respectively (Figure 2B). Vincristine alone induced DNA fragmentation in up to 35% of UW228-2 cells and in up to 63% of DAOY cells. As in the PI uptake analyses, a protective effect of IR against vincristine-mediated cytotoxicity was observed. When the UW228-2 cells were exposed to a dose of 40 Gy, DNA fragmentation was decreased to 10-12% in the vincristine pretreated cells (p<0.05 at 10 nM vincristine and p<0.005 at 30 nM vincristine). In the DAOY cells, IR of 10 or 20 Gy reduced DNA fragmentation to 25% or 20%, respectively (p<0.05 at 30 nM vincristine). As noted above, IR alone caused a significant increase of cells in the G₂/M phase. However, in contrast to HDIs, vincristine did not interfere with the IR-induced G₂/M arrest.

Discussion

Overall survival rates have shown that replacing full-dose IR by reduced-dose IR plus chemotherapy is a feasible approach (18, 19), however reduced-dose IR is still associated with significant neurological toxicity (3, 4). A further reduction in IR dose is highly desirable in order to decrease IR-related morbidity, however this might risk a reduction in disease control.

In a previous study with the medulloblastoma cell line, DA0Y, a dose of 20 Gy after pretreatment with almost nontoxic doses of HDIs was as effective in cell killing as a dose of 40 Gy without pretreatment. In the current study, the HDIs also increased the killing efficiency of IR in a second medulloblastoma cell line, UW228-2, as evidenced by PI uptake and by DNA fragmentation. These results, thus, strengthen the suggestion that HDIs may have potential as radioenhancers for the treatment of medulloblastoma. A favourable interaction of HDIs and IR has also been demonstrated for a variety of other malignancies, such as prostate cancer, squamous carcinoma, gastrointestinal cancer, glioblastoma, melanoma, breast cancer, lung cancer, and colorectal cancer (15, 16, 20-30).

Although several adjuvant chemotherapeutic regimens have been explored for medulloblastoma treatment, there is no clear best option on the ground of clinical trials (1, 31). However, vincristine, a potential radioenhancer is commonly used in radiochemotherapy treatment protocols. In both the cell lines examined in this study, vincristine did not enhance IR-induced cell death, instead, the combination of vincristine and IR resulted in significantly less cytotoxicity than vincristine alone. Especially at the higher doses, IR was found to completely abolish the cytotoxic effect of vincristine. This observation is in accord with a study on breast cancer and epidermoid carcinoma cell lines, which has demonstrated an antagonistic interaction between IR and vincristine or vinblastine (11). A lack of synergism with IR in a variety of carcinoma cell lines has also been shown for paclitaxel, another antineoplastic agent that induces G₂/M arrest (32, 33). Thus, the use of antimitotic drugs in combination with IR may merit reconsideration.

The results presented by Sui et al. indicate that the unfavourable interaction of antimitotic drugs and IR is cell cycle-dependent (11, 33). However, not only vinca alkaloids and taxanes are known to arrest cells in the G₂/M phase of the cell cycle, but also HDIs have been shown to be capable of inducing G₂/M arrest, particularly when their apoptosis-inducing activity is blocked by caspase inhibitors (34, 35). Likewise, the cell cycle analyses of UW228-2 cells in the present study revealed a trend towards an increase of cells in the G₂/M phase after exposure to either HDIs (Figure 1B) or vincristine (Figure 2B). The cell cycle analyses also revealed a distinct difference between the two treatment modalities, while pretreatment with vincristine did not interfere with IR-induced G₂/M arrest, pretreatment with
Figure 2. IR antagonises vincristine. Medulloblastoma cells were irradiated 4 h after treatment with vincristine and cultured for further 24 h (DAOY) or 48 h (UW228-2). (A) Cytofluorometric analysis of PI uptake. (B) Cytofluorometric cell cycle analysis. Means±SD of 3 experiments are shown (*p<0.05, **p<0.005).
HDDIs abolished the latter. This observation suggests that IR, even when applied subsequent to vincristine as in our study, prevents vincristine-induced cell death by arresting cells in the G2/M phase, whereas HDDIs convert IR-mediated G2/M arrest into cell killing. The explanation for the opposite effects of HDDIs and vincristine may lie in the different mechanisms by which they induce G2/M arrest. Vincristine causes a mitotic block by inhibition of mitotic spindle function (36). The mechanism by which HDDIs arrest cells in G2/M phase is less clearly defined (37). Nonetheless, HDDI-induced G2/M arrest has been found to be associated with the reduced expression of cyclins A and B as well as CDK1, key components for G2/M transition, and the increased expression of the CDK inhibitors p21 and p27 (38, 39). In addition, it has been reported that HDDIs induce a G2/M arrest by activating the p38 MAPK checkpoint (40). Hence, block of G2/M transition, as caused by HDDIs, appears to enhance the cytotoxic effect of IR, while an IR-induced G2 arrest seems to be detrimental to the anticancer activity of vincristine (or paclitaxel), as has already been suggested elsewhere (11, 33).

In conclusion, our results indicate a favourable interaction of IR and HDDIs, but an unfavourable one of IR and vincristine, in medulloblastoma cells. Given the importance of IR for the treatment of medulloblastoma and the basic necessity to develop radiochemotherapy strategies that maximise therapeutic efficacy and, at the same time, minimise normal tissue toxicity, this finding may be relevant in a clinical setting.

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References


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