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The histone deacetylase inhibitor vorinostat induces calreticulin exposure in childhood brain tumour cells in vitro

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Abstract

Purpose It has recently been recognised that anticancer chemotherapy can elicit an immunogenic form of apoptosis characterised by the exposure of calreticulin (CRT) on the surface of dying tumour cells, entailing an immune response that contributes to the therapeutic outcome. CRT exposure has been found to be induced by anthracyclins and oxaliplatin, but not by other proapoptotic antineoplastic agents including etoposide, camptothecin and cisplatin. In this study, we examined the histone deacetylase inhibitor vorinostat for its capability to stimulate CRT exposure in tumour cells.

Methods Childhood tumour cells, i.e. the brain tumour cell lines PFSK and DAOY and the Ewing's sarcoma cell line CADO-ES-1, were treated with vorinostat, and CRT exposure was determined by flow cytometric analysis of CRT immunofluorescence. Combination effects of vorinostat/TRAIL and vorinostat/bortezomib were also assessed.

Results Vorinostat treatment induced CRT exposure in PFSK and DAOY cells, but not in caspase-8-deficient CADO-ES-1 cells. CRT exposure could be prevented by the pan-caspase inhibitor z-VAD-fmk and by brefeldin A, an inhibitor of Golgi-mediated transport.

Conclusion Vorinostat has the capacity to elicit CRT exposure, suggesting its usefulness as immunogenic antitumour agent.

Keywords Bortezomib · Calreticulin · Childhood brain tumour · Histone deacetylase inhibitor · TRAIL · Vorinostat

Abbreviations

CRT Calreticulin
ER Endoplasmatic reticulum
HDACi Histone deacetylase inhibitor
SPNET Supratentorial primitive neuroectodermal tumour

Introduction

Histone deacetylase inhibitors (HDACi), such as vorinostat (suberoylanilide hydroxamic acid, SAHA), are an emerging class of promising new anticancer agents. They induce apoptosis, growth arrest and differentiation in tumour cells while leaving healthy cells relatively unscathed [1]. Their clinical potential is underscored by the fact that vorinostat has been approved by the US Food and Drug Administration for treatment of cutaneous T-cell lymphoma [2].

Engagement of antitumour immunity is another promising option to improve the management of malignant disease [3]. Although antineoplastic chemotherapy has long been regarded to induce an immunologically silent or even immunosuppressive response, recent evidence suggests that chemotherapeutic drugs can in fact have immunostimulatory properties. For example, the proteasome inhibitor bortezomib promotes the exposure of heat shock protein 90 on the surface of dying myeloma cells, which leads to the maturation of dendritic cells allowing the latter to activate T cells [4].

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Calreticulin (CRT) is another protein whose exposure on the plasma membrane of apoptotic cells can result in the activation of immune cells [5, 6]. CRT usually resides in the endoplasmic reticulum (ER); chemotherapy, however, may cause apoptotic cancer cells to translocate CRT to their surface. The presence of CRT on the surface of tumour cells then directs their uptake by dendritic cells that induce an antitumour immune response [6]. But among the cytotoxic agents tested, only few—the anthracyclins and oxaliplatin—induce CRT translocation and in turn immunogenic cell death, whereas others—e.g. mitomycin C, etoposide, camptothecin and cisplatin—do not [6, 7].

HDACi can potentially also stimulate an anticancer immune response. For instance, they have been shown to induce tumour cell-surface expression of NKG2D ligands and boost tumour cell recognition and lysis by NK cells [8–10], to upregulate the expression of MHC class I and class II proteins [11] as well as of costimulatory molecules [12], and to enhance antigen processing and presentation [13]. However, it has not yet been studied whether HDACi cause CRT translocation and, this way, an immunogenic activity.

To explore this issue, we investigated whether vorinostat could induce CRT exposure in childhood brain tumour cells. Brain tumours are the most common solid malignancies of childhood and a leading cause of cancer-related death in children. Despite advances in therapy of childhood brain tumours in general over the last few decades, the prognosis remains dismal for a subset of patients, particularly those with disseminated disease [14]. In addition, current therapies are still frequently associated with severe long-term sequelae. Thus, the development of treatment strategies that may improve the chance of survival as well as lessen side effects is of paramount importance. The utilisation of the immune system to target brain tumours may be a valuable option [15].

We examined a possible CRT-exposing activity of vorinostat in two paediatric brain tumour cell lines: the supratentorial primitive neuroectodermal tumour (sPNET) cell line PFSK and the medulloblastoma cell line DAOY. We have recently shown that vorinostat induces apoptosis in these cell lines [16, 17]. In the present report, we show that vorinostat treatment also elicits CRT exposure.

Materials and methods

Reagents

Vorinostat, mitomycin C, brefeldin A and z-VAD-fmk were purchased from Alexis (Grünberg, Germany). Bortezomib was purchased from LC Laboratories (Woburn, MA, USA). TRAIL was purchased from Peprotech (Hamburg, Germany).

Cell lines

DAOY and PFSK cells were a gift from Dr. M. Grotzer (Zurich, Switzerland) and maintained in Improved MEM Zinc Option (Invitrogen, Karlsruhe, Germany) or RPMI 1640 (PAA, Cölbe, Germany), respectively. CADO-ES-1 cells were obtained from the DSMZ (Braunschweig, Germany) and maintained in RPMI 1640. Media were supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin G sodium and 100 µg/ml streptomycin sulphate (PAA). CADO-ES-1 cells were cultivated in collagen-coated (5 µg/cm²; Roche, Mannheim, Germany) tissue culture flasks. Cells were cultivated at 37°C in a humidified 5% CO₂ incubator and routinely passaged when 90% confluent. Cell viability was determined by the trypan blue exclusion test. Cells were regularly inspected to be free of mycoplasma with the PCR mycoplasma detection kit from AppliChem (Darmstadt, Germany).

Flow cytometric detection of cell-surface CRT

The cells were plated at 1×10^5 cells in 12-well plates and treated with vorinostat for 24 h. Brefeldin A or z-VAD-fmk was applied 1 h before treatment; in the combination experiments, vorinostat was applied 4 h before TRAIL or bortezomib. Cells were harvested with 0.2% EDTA, washed twice with PBS, and incubated with the mouse anti-CRT monoclonal antibody FMC 75 (2 µg in 100 µl PBS/2.5% BSA; Abcam, Cambridge, UK) for 45 min at 4°C in the dark. Cells were washed twice and stained with a FITC-conjugated goat anti-mouse IgG antibody (20 µg/ml in 2.5% BSA; Dianova, Hamburg, Germany) for 45 min at 4°C in the dark. After washing, 50,000 cells were analysed using a BD (Heidelberg, Germany) FACSCanto II; cells were gated on viable cells. A mouse IgG (20 µg/ml in 2.5% BSA; eBioscience, San Diego, CA, USA) was used as isotype-matched control. Frequency histograms were generated using Flowjo software (Ashland, OR, USA). Data are shown as fold induction after calculation of the mean fluorescence intensities of anti-CRT-stained cells divided by isotype-matched IgG-stained cells.

Immunoblot detection of total CRT

After a 24-h incubation with vorinostat, cells were lysed on ice for 15 min in 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS supplemented with a protease inhibitor cocktail (Roche) followed by brief sonification. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Concentration of 30 µg of total cellular protein per lane was separated by standard SDS-PAGE on 10% gel and

electrophoretically transferred to nitrocellulose membrane (Whatman, Dassel, Germany). After blocking in PBS containing 5% dry milk and 0.05% Tween 20, CRT was immunodetected using FMC 75 antibody (dilution 1:1,000). Equal loading of protein was verified by detection of GAPDH using mouse anti-GAPDH monoclonal antibody (dilution 1:10,000; Biodesign International, Saco, ME, USA). Peroxidase-conjugated goat anti-mouse IgG (dilution 1:25,000; Dianova) was used as secondary antibody followed by enhanced chemiluminescence detection (Amersham, Freiburg, Germany) of specific signals.

Statistical analysis

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

Results

CRT exposure on PFSK sPNET cells

To test whether vorinostat elicits CRT exposure in paediatric brain tumour cells, we initially determined CRT surface expression on PFSK cells. Figure 1a, b demonstrates that vorinostat induced CRT exposure in a dose-dependent manner, as monitored by flow cytometric analysis of viable cells for CRT immunofluorescence staining. To exclude a passive leakage of CRT to the plasma membrane, cells were pretreated with brefeldin A, an inhibitor of protein transport from the ER to the Golgi apparatus: Inhibition of ER–Golgi traffic abolished vorinostat-elicited CRT exposure. Because chemotherapy-mediated CRT translocation has been shown to involve caspases [6], PFSK cells were pretreated with the pan-caspase inhibitor z-VAD-fmk: Caspase inhibition prevented the CRT-exposing activity of vorinostat.

CRT exposure on DAOY medulloblastoma cells

Next, we studied the effect of vorinostat on CRT exposure in DAOY cells. As shown in Fig. 1c, vorinostat treatment also provoked a concentration-dependent CRT surface expression in these cells. As a negative control, DAOY cells were also treated with mitomycin C, which has been reported to exert no CRT-exposing effect [6]. Mitomycin C did not increase CRT exposure in DAOY cells. In addition, we examined whether CRT exposure in vorinostat-treated cells was accompanied by a general rise of CRT abundance. Immunoblot detection of CRT in total cell lysates showed that vorinostat treatment did not result in an increase in total CRT (Fig. 1d).

CRT exposure on CADO-ES-1 Ewing's sarcoma cells

Caspase-8 has been shown to be necessary for anthracycline- and oxaliplatin-induced CRT exposure [18]. To shed light on the relevance of caspase-8 for HDACi-induced CRT exposure, we employed the Ewing's sarcoma cell line CADO-ES-1, a cell line deficient in caspase-8 expression [19], but apoptotically responsive to HDACi treatment [20]. Figure 1e illustrates that vorinostat had no effect on CRT surface expression on CADO-ES-1 cells.

CRT exposure after vorinostat/TRAIL or vorinostat/bortezomib combination treatment

A large number of preclinical studies have demonstrated synergistic proapoptotic activity of vorinostat with other antineoplastic agents, such as the death receptor ligand TRAIL or the proteasome inhibitor bortezomib [2]. We thus evaluated whether vorinostat could also synergise with TRAIL or bortezomib in triggering CRT exposure. In consistency with the observation that TRAIL elicits CRT exposure in HeLa cells [18], we found that TRAIL evoked CRT surface expression on DAOY cells (Fig. 2a). Yet the vorinostat/TRAIL combination treatment produced a merely additive effect. However, when vorinostat was combined with bortezomib, a trend to overadditive induction of CRT exposure was observed (Fig. 2b).

Discussion

To the best of our knowledge, we herein report for the first time that treatment with an HDACi leads to CRT surface exposition on tumour cells. Given the limited number of chemotherapeutics with proven CRT-exposing activity—the anthracyclins and oxaliplatin [6, 7]—this entails a substantial expansion of antineoplastic agents endowed with the potential to elicit a CRT-dependent antitumour immune response.

In our study, we have shown that vorinostat induced CRT exposure in a concentration-dependent fashion. Several lines of evidence presented indicate that vorinostat promotes CRT exposure by the same mechanism as the known CRT-exposing compounds. First, we could not detect a rise of total CRT expression, in concordance with the finding that exposure of CRT induced by anthracyclins does not stem from a general increase in CRT abundance [6]. Second, CRT translocation has been demonstrated to occur via the ER–Golgi pathway [18]. Our results show that the vorinostat-mediated CRT exposure could be prevented by brefeldin A, suggesting that CRT translocation induced by vorinostat also depends on anterograde ER–Golgi traffic. Third, in line with the observation that chemotherapy-elicited

Fig. 1 Induction of CRT exposure by vorinostat. Cells were treated with vorinostat for 24 h. **a, b, c, e**, CRT cell-surface expression was determined by flow cytometric analysis of viable cells. **a**, Cells were stained with CRT antibody (*solid line*) or isotype control (*filled histogram*). **b, c, e**, CRT mean fluorescence is depicted as fold induction after calculation of the mean fluorescence intensities of anti-CRT-stained cells divided by isotype-matched IgG-stained cells; mean \pm SEM. of each 3 experiments are shown (*treatment vs. control; #brefeldin A and z-VAD-fmk, respectively, vs. 5 μ M vorinostat). **d**, total CRT expression was determined by immunoblot analysis of cell lysates

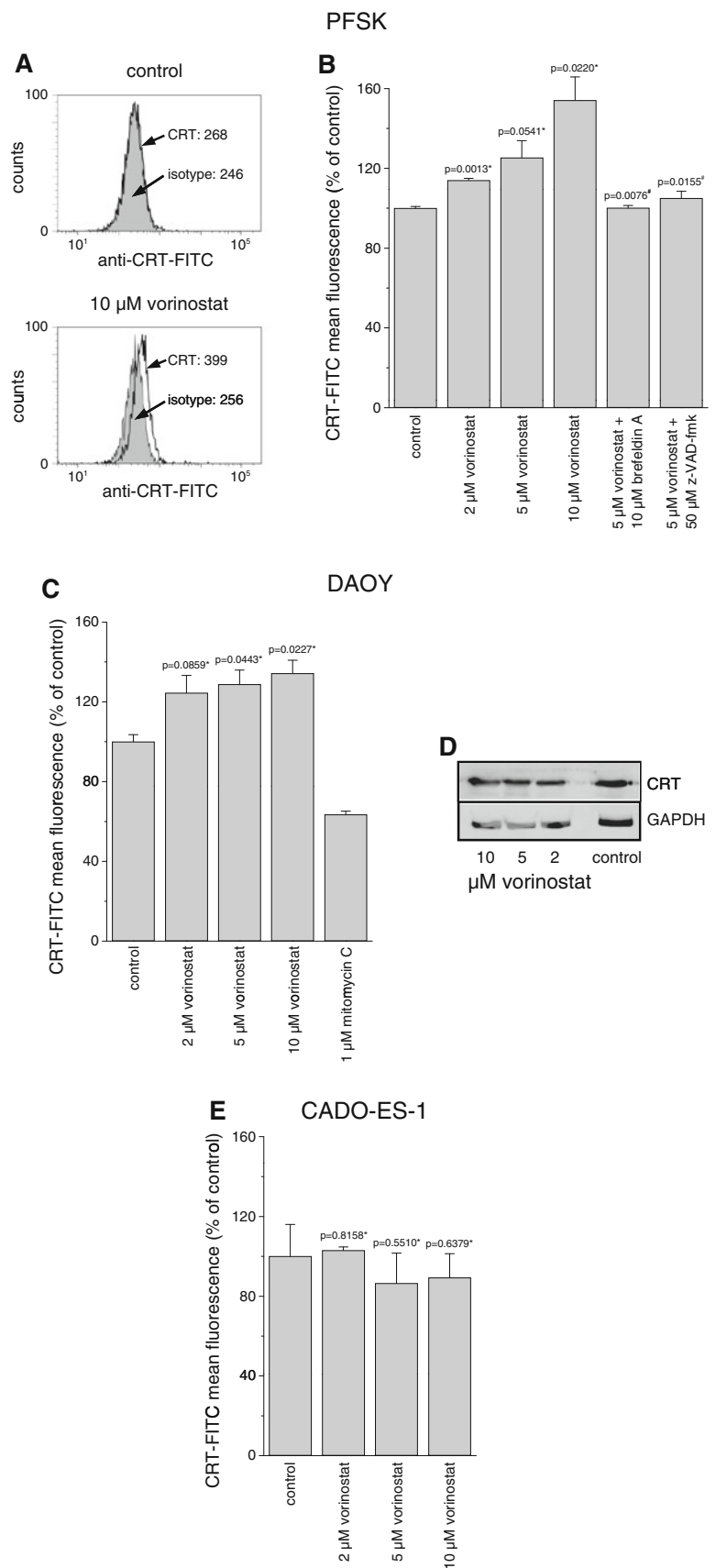
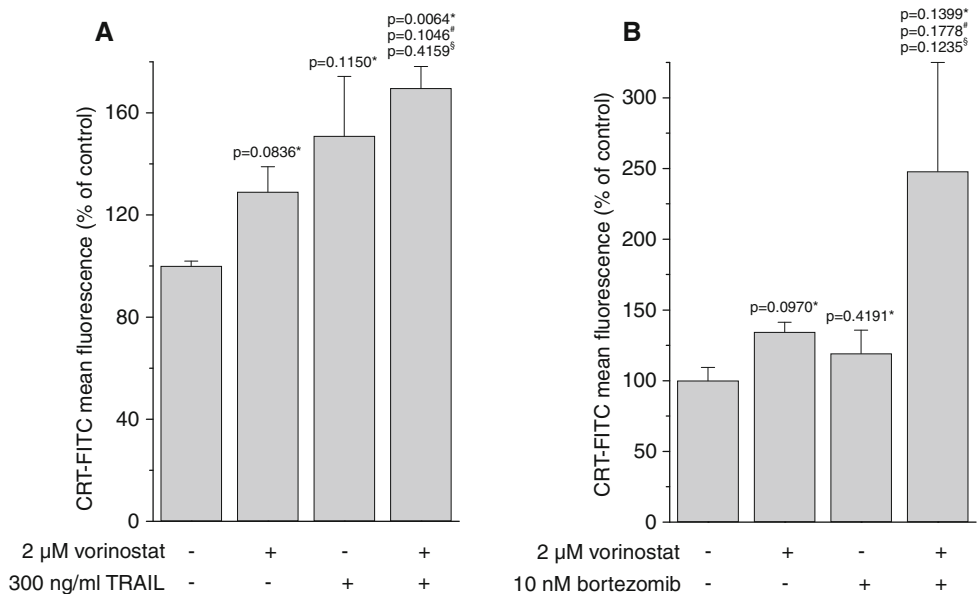


Fig. 2 Induction of CRT exposure by vorinostat in combination with TRAIL or bortezomib. 4 h after administration of vorinostat, DAOY cells were treated with TRAIL (a) or bortezomib (b) for 24 h. CRT cell-surface expression was determined by flow cytometric analysis of viable cells. CRT mean fluorescence is depicted as fold induction after calculation of the mean fluorescence intensities of anti-CRT-stained cells divided by isotype-matched IgG-stained cells; mean \pm SEM. of each 3 experiments are shown (*treatment vs. control; #combination treatment vs. vorinostat; §combination treatment vs. TRAIL or bortezomib, respectively)



CRT surface expression depends on caspase activity [6], vorinostat-induced CRT exposure could be abolished by z-VAD-fmk. In addition, vorinostat failed to promote CRT exposure in caspase-8-deficient CADO-ES-1 cells—which are otherwise susceptible to HDACi-treatment [20]—consistent with the finding that caspase-8 is necessary for CRT exposure, but not apoptosis, induced by anthracyclins and oxaliplatin [18].

A multitude of preclinical studies have documented that HDACi have great potential to augment the anticancer efficiency of other therapeutic regimens, such as chemo- and radiotherapy [1, 2]. With respect to the use of TRAIL for the treatment of childhood tumours, we have previously shown that vorinostat and TRAIL synergise to elicit cell death in medulloblastoma, Ewing's sarcoma and hepatoblastoma [17, 20, 21]. TRAIL seemed furthermore to be particularly interesting since it has been demonstrated to stimulate CRT exposure in carcinoma cells [18]. In the attempt to find out whether vorinostat and TRAIL synergised in inducing CRT exposure, however, we detected a merely additive effect. In contrast, the combination of vorinostat with bortezomib—the latter has been reported to elicit immunogenic cell death by inducing exposure of heat shock protein 90 [4]—produced a trend to enhanced induction of CRT surface expression. These findings show that the combination of two CRT-exposing compounds does not necessarily result in the potentiation of CRT exposure, but they also show that it can in principle be augmented by a suitable combination treatment.

Again to the best of our knowledge, we here have also shown for the first time that childhood brain tumour cells can respond to antineoplastic agents by exposing CRT. Therefore, our results suggest that the concept of immunotherapy [3] may be a therapeutic option in these

difficult to treat tumours. Chemotherapy of brain tumours is in general complicated in that the blood–brain barrier obstructs the penetration of most drugs into the brain [22]. However, this shortcoming does supposedly not apply to vorinostat, for it has been shown to cross the blood–brain barrier in mice [23]. To sum up, our study on the one hand extends the notion that HDACi have immunomodulatory activity that may contribute to mediating their antitumour effects [8–13] and on the other supports their potential usefulness in paediatric oncology [24].

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