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Histone deacetylase inhibitors sensitize tumour cells for cytotoxic effects of natural killer cells

Mareike Schmudde [a,*], André Braun [a], Daniela Pende [b], Jürgen Sonnemann [c], Ulrike Klier [a], James F. Beck [d], Lorenzo Moretta [b], Barbara M. Bröker [a]

[a] *Institut für Immunologie und Immunologie, Ernst-Moritz-Arndt-Universität Greifswald, Ferdinand-Sauerbruch-Str., D-17487 Greifswald, Germany*
[b] *Istituto Nazionale per la Ricerca sul Cancro, Università degli Studi di Genova, Genoa, Italy*
[c] *Research Center of Pharmacology and Experimental Therapeutics, Ernst Moritz Arndt University Greifswald, Greifswald, Germany*
[d] *Department of Pediatric Hematology and Oncology, University Children's Hospital Jena, Jena, Germany*

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Abstract

Histone deacetylase inhibitors (HDIs) are emerging as potent anti-tumour agents which induce cell cycle arrest, differentiation and/or apoptosis in many tumour cells. Furthermore, they render tumour cells more sensitive to other therapeutic regimens like ionizing radiation, chemotherapy and recombinant tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Here, we show that the HDIs suberoylanilide hydroxamic acid (SAHA; vorinostat), sodium butyrate (NaB) and MS-275 sensitized DAOY and PC3 tumour cells for the cytotoxic effects of IL-2-activated PBMCs. In ^{51}Cr-release assays, blockade of the activating NK receptors DNAM-1, NKG2D and the NCRs completely abrogated tumour cell lysis, revealing that NK cells were the main effector cells involved. HDIs increased the tumour surface expression of ligands for the activating NK receptors NKG2D and DNAM-1 thereby facilitating tumour cell recognition by NK cells. These results suggest that the combination of HDIs and immunotherapy may be an effective strategy for anti-cancer therapy.

Keywords: Histone deacetylase inhibitors; NK cells; SAHA; Sodium butyrate; Synergy

1. Introduction

Histone deacetylase inhibitors (HDIs) are emerging as potent anti-tumour agents. They inhibit the action of histone deacetylases, thereby leading to a higher acetylation status of the chromatin. This results in an increase in transcriptionally active chromatin. By influencing the transcription of up to 22% of genes [1], HDIs induce cell cycle arrest, differentiation and/or apoptosis in tumour cells of many different histological origins. Notably, they affect cancer cells while leaving their untransformed counterparts largely unimpaired [2,3]. Several HDIs are currently tested in phase I and II trials and they have yielded favourable

* Corresponding author. Tel.: +49 3834865466; fax: +49 3834865490.
E-mail address: mareike.schmudde@uni-greifswald.de (M. Schmudde).

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results for the treatment of hematological malignancies as well as solid tumours [4–8]. Recently, SAHA has been approved by the US Food and Drug Administration for treatment of cutaneous T cell lymphoma [9].

Beside their direct anti-tumour activity, HDIs also enhance the cytotoxic effects of other therapeutic regimens like ionizing radiation, chemotherapy and recombinant tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) [10–13]. Since TRAIL is an important effector molecule of cytotoxic immune cells the question arose whether HDIs can sensitize tumour cells for the cytolytic effects of the immune system. Two main immune cell populations are involved in the defence against tumours. Tumour specific $CD8^+$ T cells of the adaptive immune system recognize tumour antigens and, if given a co-stimulatory signal, differentiate to cytotoxic T lymphocytes. On the other hand, natural killer (NK) cells are the main cytotoxic effector cells of the innate immune system. In contrast to T lymphocytes, NK cells do not express specific antigen receptors but their action is regulated by various activating and inhibitory receptors. Inhibitory signals are transmitted to an NK cell upon binding of KIRs to HLA class I molecules and/or CD94/NKG2A heterodimers to HLA-E [14]. NK cell activation depends on the engagement of triggering receptors of which the most important are NKG2D [15], DNAM-1 [16] and the "natural cytotoxicity receptors" (NCRs) NKp46, NKp44 and NKp30 [17]. While expression of the NCRs is restricted to NK cells, NKG2D is also expressed by γ/δ^+ T cells and α/β $CD8^+$ T cells.

Human NKG2D ligands are represented by MICA and MICB and the family of UL16-binding proteins (ULBPs) which are structurally related to MHC class I molecules. NKG2D ligands are up-regulated in various tumour cells and can be induced in untransformed cells upon stress [18,19]. The DNAM-1 ligands PVR (polio virus receptor, CD155) and Nectin-2 (CD112) may be over-expressed on tumour cells of different histological origins [20]. The ligands for the NCRs are not known so far.

In the present study, we assessed the impact of HDI pre-treatment on tumour cell recognition and lysis by human IL-2-activated PBMCs. We show that HDI treatment up-regulates the surface expression of NKG2D ligands on tumour cells and sensitizes them for the cytotoxic effects of NK cells.

2. Materials and methods

2.1. Cell culture

PC3 prostate carcinoma cells were obtained from ATCC (Rockville, MD, USA) and maintained in DMEM, supplemented with 10% foetal calf serum, 4 mM L-glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin. DAOY medulloblastoma cells were a gift from Dr. M. Grotzer (Zurich, Switzerland) and maintained in Improved MEM Zinc Option, supplemented with 10% foetal calf serum, 200 U/ml penicillin and 200 μg/ml streptomycin. NK92C1 were from ATCC and maintained in MEM-α supplemented with 12% foetal calf serum, 12% donor horse serum, 200 U/ml penicillin and 200 μg/ml streptomycin. PBMCs were isolated from buffy coats of healthy donors by Ficoll–Hypaque density gradient centrifugation and cultured for 3 days in RPMI supplemented with 10% human serum from healthy donors, 4 mM L-glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin and 6000 U/ml rIL-2. NK cells from peripheral blood of healthy donors were isolated using the RosetteSep method (StemCell Technologies, Vancouver, BC, Canada). NK cells were cultured on irradiated feeder cells in the presence of 2 μg/ml phytohemagglutinin and 100 U/ml rIL-2 to obtain proliferation of polyclonal NK cell populations. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Reagents and antibodies

SAHA and MS-275 were purchased from Alexis (Grünberg, Germany), NaB, vincristine, phytohemagglutinin and monensin were purchased from Sigma (Deisenhofen, Germany) and rIL-2 (Proleukin) was purchased from Chiron (Emeryville, CA, USA). Fixation Medium was obtained from ADG (Kaumberg, Austria) and RNase A was obtained from Roche Diagnostics (Mannheim, Germany). Anti-MICB monoclonal antibody (mAb) was from R&D Systems (Wiesbaden, Germany), anti-TRAIL mAb was from Abcam (Cambridge, UK), anti-DR4 and anti-DR5 mAbs were from Biozol (Eching, Germany). Anti-MHC class I (clone W6-32) was from a hybridoma obtained from ATCC and grown in the laboratory. Murine IgG1 isotype control antibody was from eBioscience (San Diego, CA, USA), murine IgG2a isotype control was from DakoCytomation (Hamburg, Germany) and murine IgG2b

isotype control was from BD Biosciences (Heidelberg, Germany). Monoclonal CD3-PE, monoclonal CD8-FITC, monoclonal CD56-FITC, monoclonal HLA-DR-PE, monoclonal CD80-FITC, monoclonal CD86-PE, monoclonal Fas-FITC and monoclonal CD107a-PE-Cy5 were from BD Biosciences. Directly labelled murine isotype controls (IgG2a–PE, IgG1–FITC, IgG2b–PE and IgG1–PE-Cy5) and secondary polyclonal goat anti-mouse IgG/IgM–FITC were from BD Biosciences. The following mAbs, used for surface staining, were selected at the IST (Genova, Italy): BAM195 (IgG1, anti-MICA), L95 (IgG1, anti-PVR) and L14 (IgG2a, anti-Nectin-2). M295 (IgG1, anti-ULBP1), M311 (IgG1, anti-ULBP2) and M551 (IgG1, anti-ULBP3) were kindly provided by Amgen (Seattle, WA, USA). PE-conjugated isotype-specific goat anti-mouse second reagents (Southern Biotechnology Associated, Birmingham, AL, USA) were used. For masking experiments the following mAbs were used: F252 (IgM, anti-NKp30), F5 (IgM, anti-DNAM-1) and BAT221 (IgG1, anti-NKG2D) were selected at the IST; KS38 (IgM, anti-NKp44) and KL247 (IgM, anti-NKp46) were kindly provided by S. Parolini (University of Brescia, Brescia, Italy).

2.3. Measurement of cell death

Tumour cells were pre-treated with HDIs or vincristine for 24 h. After changing the culture medium to avoid immunosuppressive effects of the HDIs, cells were exposed to IL-2-activated human PBMCs or IL-2-activated NK92C1 cells. Tumour cell death was then assessed by counting surviving tumour cells. After 24 or 48 h, adherent tumour cells were harvested using trypsin/EDTA and cells were counted using a FACScan gating on the tumour cells. Absolute cell numbers were calculated by adding 50,000 microbeads to each sample. Dead cells were excluded by propidium iodide staining. The results were analyzed by the combination index (CI) method according to Chou and Talalay [21] using Calcusyn software from Biosoft (Cambridge, UK).

Cytotoxicity of IL-2-activated PBMCs was also analyzed in a 4-h ^{51}Cr-release assay as described previously [22]. In short, 5000 ^{51}Cr-labelled tumour cells were cultured together with IL-2-activated PBMCs at the indicated effector-to-target (E/T) ratios. After 4 h, ^{51}Cr-release was determined by counting the supernatant in a γ-counter. For masking experiments, effector cells were pre-incubated with mAbs specific for various NK receptors at concentrations of 10 μg/ml.

2.4. CD107a mobilization assay

Degranulating IL-2-activated PBMCs were identified by analysis of surface expression of CD107a. PC3 cells were cultured with or without HDIs for 24 h. After changing the medium they were incubated for 5 h at 37 °C with IL-2-activated PBMCs at an E/T of approximately 1:1 in the presence of 10 μl of anti-CD107a antibody and 10 μM monensin in a total volume of 0.5 ml. After the incubation, PBMCs were washed and stained with antibodies specific for CD107a and for the NK or T cell markers CD3 and CD56 or CD8, respectively.

2.5. Cytofluorometric analysis of surface molecules

Tumour cells were harvested 24 or 48 h after treatment with HDIs and stained with the appropriate antibodies for 40 min at 4 °C, washed and if necessary stained with a secondary antibody for 40 min at 4 °C. After washing, 50,000 cells were analyzed using a FACScan. To compare the surface expression of the molecules on treated and untreated cells, we calculated the mean relative fluorescence intensity (MRFI), that is, the ratio between the mean fluorescence intensity of cells stained with the specific mAb and that of cells stained with the isotype-matched control mouse Ig. FACS data were analyzed by WinMDI 2.8.

To correlate MICA surface expression with cell cycle phases, we analyzed propidium iodide (PI) incorporation into the DNA of MICA-stained cells. After incubation with the secondary antibody, tumour cells were fixed for 15 min using fixation medium. After washing, cells were resuspended in PBS containing 1% glucose, 50 μg/ml RNase A and 50 μg/ml PI and incubated in the dark at room temperature for 30 min. Cells (100,000) were analyzed using a FACSCalibur and WinMDI 2.8 software.

3. Results

3.1. HDIs sensitize tumour cells for cell death induction by PBMCs

DAOY and PC3 cells were exposed for 24 h to HDIs belonging to three different structural classes, the hydroxamic acid SAHA (1 and 5 μM), the short-chain fatty acid NaB (1 and 5 mM) and the benzamide

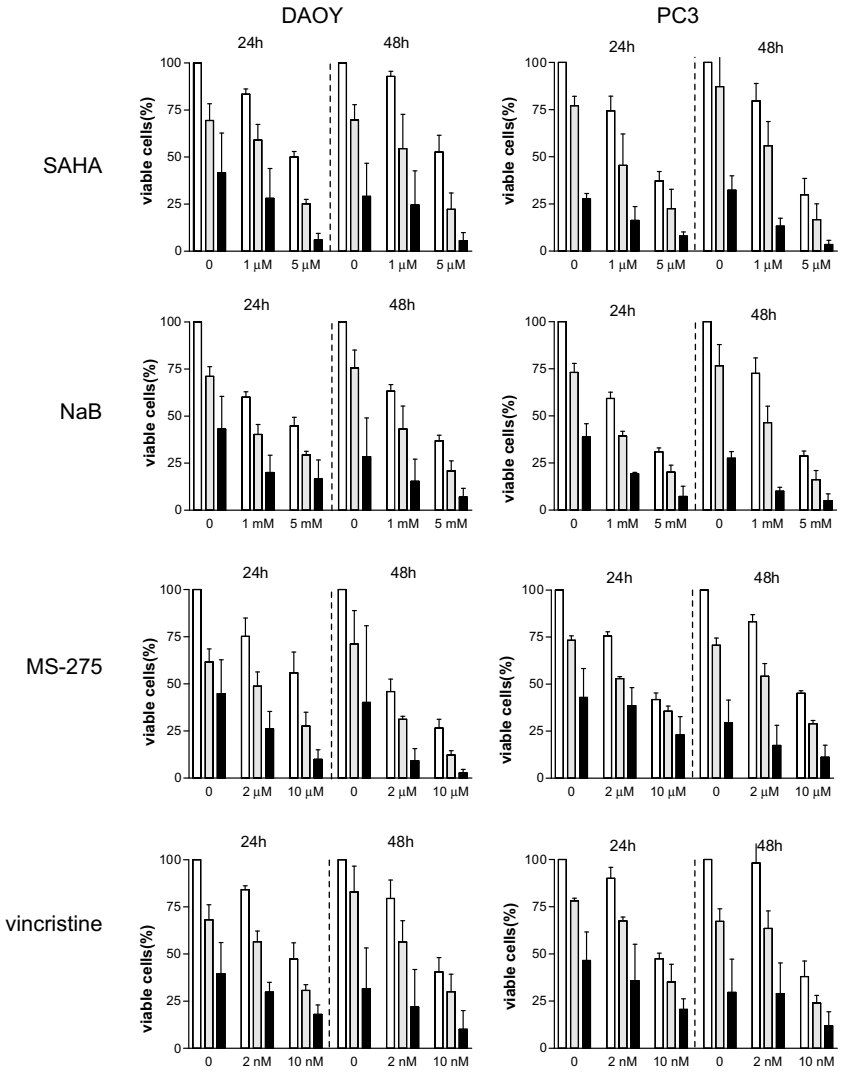

Fig. 1. Tumour cell death induction by HDIs and IL-2-activated PBMCs. HDI-pre-treated tumour cells were co-incubated with 10^5 IL-2-activated PBMCs (grey bars), 10^6 IL-2-activated PBMCs (black bars) or without PBMCs (white bars) for 24 or 48 h. Results indicate percentages of viable tumour cells (means ± SD; $n \geqslant 3$).

MS-275 (2 and 10 μM). Their effects were compared to those of the cytostatic agent vincristine (2 and 10 nM), a microtubule-targeting drug which arrests cells in metaphase. Cell death was then measured as reduction of viable cell numbers (Fig. 1). Each drug induced dose-dependent cell death in both tumour cell lines, e.g. incubation with 1 μM SAHA and 5 μM SAHA for 24 h reduced the number of viable DAOY cells to 84% and 50%, respectively. Similarly, IL-2-activated PBMCs decreased the viability of DAOY and PC3 cells in a dose-dependent manner. Twenty-four hours of incubation with 10^5/ml PBMCs, which corresponds to an E/T ratio of approximately 1:1, reduced viable DAOY cells to 69%, 10^6/ml PBMCs reduced viable DAOY cells to 42%. After 48 h the effect was even more pronounced.

Subsequently, tumour cells were pre-treated with HDIs or vincristine for 24 h before exposing them to IL-2-activated PBMCs. This combination further enhanced the loss of tumour cell viability (Fig. 1). While, for example, 24 h of incubation with 5 μM SAHA or 10^6/ml PBMCs alone reduced the viability of DAOY cells by approximately one half (50% and 42% of viable DAOY cells, respectively) the combination of cytolytic effector cells with HDI pre-treatment resulted in only 6% of viable DAOY cells. Exposure to NaB and MS-275 had similar effects, and vincristine also increased tumour cell death induction by PBMCs in DAOY and PC3 cells.

To quantify the combinatory effect of HDI/vincristine treatment and co-incubation with PBMCs we compared the combination indices (CI) for the different treatments

Table 1
Combination index values of HDIs and PBMCs

Drug	PBMC incubation (h)	Drug conc.	PBMC conc.	CI	
				DAOY	PC3
SAHA	24	1 μM	10^5/ml	0.69	0.51
			10^6/ml	0.38	0.55
		5 μM	10^5/ml	0.36	0.56
			10^6/ml	0.07	0.34
	48	1 μM	10^5/ml	0.63	0.67
			10^6/ml	0.82	0.47
		5 μM	10^5/ml	0.47	0.63
			10^6/ml	0.22	0.28
NaB	24	1 mM	10^5/ml	0.20	0.44
			10^6/ml	0.13	0.30
		5 mM	10^5/ml	0.21	0.48
			10^6/ml	0.10	0.13
	48	1 mM	10^5/ml	0.50	0.62
			10^6/ml	0.45	0.33
		5 mM	10^5/ml	0.37	0.58
			10^6/ml	0.20	0.29
MS-275	24	2 μM	10^5/ml	0.29	0.53
			10^6/ml	0.08	0.89
		10 μM	10^5/ml	0.12	0.81
			10^6/ml	0.01	0.57
	48	2 μM	10^5/ml	0.36	0.67
			10^6/ml	0.05	0.47
		10 μM	10^5/ml	0.18	0.63
			10^6/ml	0.01	0.39
Vincristine	24	2 nM	10^5/ml	0.66	0.77
			10^6/ml	0.53	0.63
		10 nM	10^5/ml	0.57	0.75
			10^6/ml	0.40	0.56
	48	2 nM	10^5/ml	0.64	1.08
			10^6/ml	0.71	1.14
		10 nM	10^5/ml	0.75	0.85
			10^6/ml	0.45	0.77

CI < 1, $= 1$ and > 1 indicate synergism, additive effect and antagonism, respectively, $n \geqslant 3$.

(Table 1). In almost all cases the CI values for treatment with HDIs were below the values for treatment with vincristine. In DAOY cells, the combination of 24 h exposure to 10^5/ml PBMCs with pre-treatment with 1 μM SAHA resulted in a CI value of 0.69. When DAOY cells had been pre-treated with 5 μM SAHA and then exposed to 10^6/ml PBMCs the CI value decreased to 0.07, indicating a very strong synergism. Using NaB or MS-275, the CI values pointed to an even stronger synergism. On the other hand, vincristine showed a much lower synergistic action with PBMCs. Combination of 10 nM vincristine with 10^6/ml PBMCs resulted only in a CI value of 0.4. For PC3 cells the findings were similar, except that the sensitizing effect of the HDI MS-275 was as low as that of vincristine. In both cell lines, the synergisms of almost all examined HDIs decreased when the tumour cells were exposed to PBMCs for 48 h.

To assess the short-term effects of HDI treatment on tumour cell death induction by PBMCs we additionally performed ^{51}Cr-release assays where tumour cells treated with 5 μM SAHA (data not shown) or 5 mM NaB (Fig. 2) as target cells were co-incubated with IL-2-activated PBMCs as effector cells for 4 h. Cell lysis of HDI-treated cells was slightly higher than that of untreated tumour cells at same E/T ratios. However, the HDI-effect was lower than after 24 or 48 h (Fig. 1).

3.2. NK cells are activated by co-incubation with tumour cells

To identify the effector cells responsible for tumour cell death in our system we assessed degranulation by cell surface staining of CD107a. CD107a is mobilized to the cell surface of activated NK cells and $CD8^+$ T cells. These cells release the contents of their cytotoxic granules into the intercellular space when their cytotoxic effector functions are triggered by contact with their target cells. This is accompanied by the mobilization of CD107a from the intracellular granules to the cell surface [23,24]. We therefore used this marker to study the activation status of the different PBMC populations after 5 h of co-incubation with tumour cells in the presence of monensin and CD107a antibody. $CD3^-CD56^+$ NK cells and $CD3^+CD8^+$ T cells were assessed separately. PBMCs which had no contact to PC3 cells expressed almost no CD107a (5% of NK cells, 1% of $CD8^+$ T cells) (Fig. 3). However, 46% of NK cells mobilized CD107a to the cell

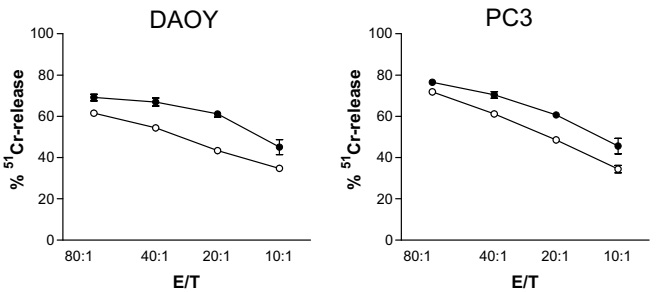

Fig. 2. Short-term tumour cell death induction by IL-2-activated PBMCs. ^{51}Cr-labelled NaB-treated (5 mM; black circles) or untreated (open circles) tumour cells were used as target cells for IL-2-activated PBMCs at indicated E/T ratios. (means ± range of duplicates).

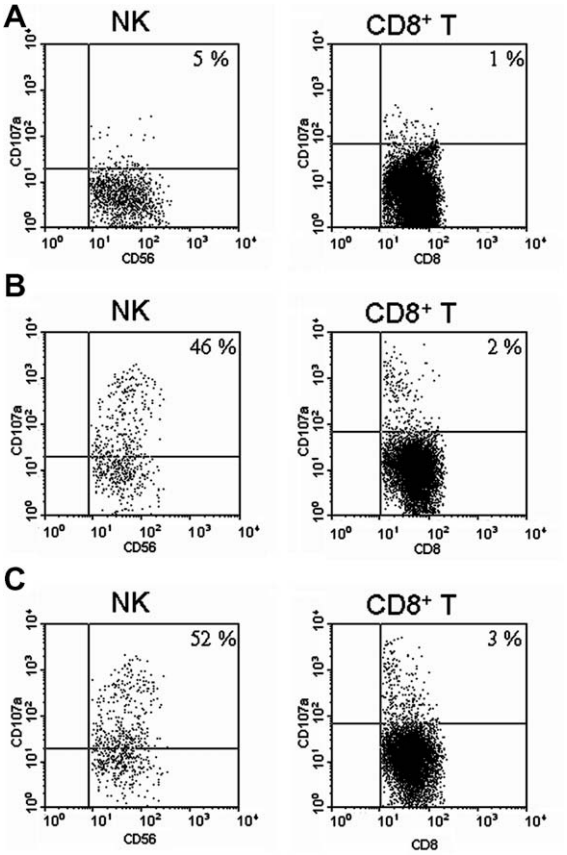

Fig. 3. Mobilization of CD107a on NK cells and $CD8^+$ T cells. (A) PBMCs had no contact to PC3 cells. (B) PBMCs were co-incubated for 5 h with untreated PC3 cells. (C) PBMCs were co-incubated for 5 h with PC3 cells pre-treated with 5 μM SAHA.

surface after co-incubation with PC3 cells. In contrast, surface expression of CD107a remained low in $CD8^+$ T cells (2%). This indicates that NK cells are the main cytolytic effector cells in our system. When we used PC3 cells treated with 5 μM SAHA (Fig. 3) or 5 mM NaB (data not shown) as stimulators the percentages of CD107a positive NK cells and $CD8^+$ T cells did not further increase (52% of NK cells, 3% of $CD8^+$ T cells).

3.3. HDIs enhance cell death induction by NK92C1 cells in DAOY cells

To confirm that NK cells can act as effector cells in our system we tested the NK cell line NK92C1. HDI-treated or untreated DAOY cells were exposed to NK92C1 cells which had been pre-activated with rIL-2 similar to the PBMCs, and after 24 or 48 h the percentage of viable tumour cells was measured (Fig. 4). Using untreated DAOY cells, 24-h incubation with 10^4/ml and 10^5/ml IL-2-activated NK92C1 cells reduced the number of viable tumour cells to 95% and 52%, respectively. Tumour cell death was strongly enhanced when the tumour cells

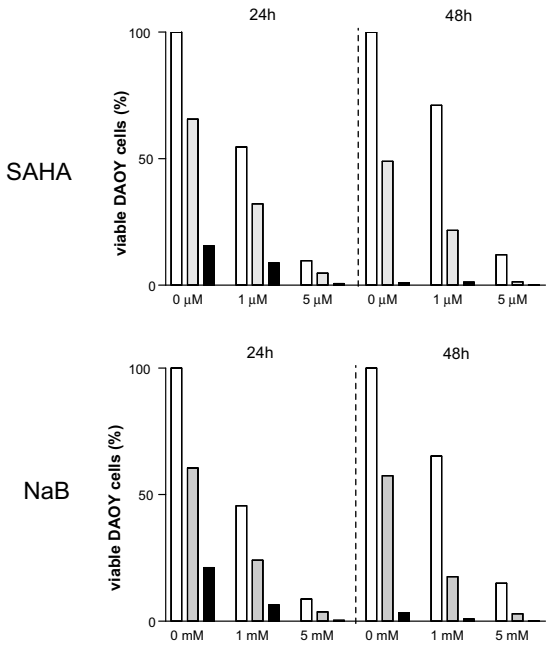

Fig. 4. Tumour cell death induction by HDIs and IL-2-activated NK92C1 cells. HDI-pre-treated DAOY cells were co-incubated with 10^4 IL-2-activated NK92C1 cells (grey bars), 10^5 IL-2-activated NK92C1 cells (black bars) or without NK92C1 cells (white bars) for 24 or 48 h. Results indicate percentages of viable tumour cells (one of two independent experiments with similar results).

had been pre-treated with SAHA or NaB. Treatment with 1 μM SAHA and co-incubation with 10^5/ml NK92C1 cells for 24 h led to 28% of viable DAOY cells. Only 5% of viable DAOY cells could be detected after pre-treatment with 5 μM SAHA and co-incubation with 10^5/ml NK92C1 cells for 24 h. Forty-eight hours of incubation with NK92C1 cells resulted in an even stronger reduction of viable tumour cells. Both SAHA and NaB had strong effects sensitizing DAOY cells for the cytolytic effector functions of NK92C1. This is clearly shown by the CI values for the combination of 10^5/ml NK92C1 with 5 μM SAHA or 5 mM NaB which are consistently below 0.4 (Table 2).

3.4. Lysis of tumour cells by PBMCs depends on activating NK receptors

Since NK cells were activated by DAOY and PC3 cells and HDIs enhanced tumour cell lysis by NK92C1 cells and by IL-2-activated PBMCs, we next examined the role of activating NK receptors in the recognition of these tumour cells. Therefore, we compared the cytolytic activity of IL-2-activated PBMCs either in the absence or presence of blocking monoclonal antibodies against the major known activating NK receptors NKG2D, DNAM-1 and the NCRs. The masking of receptors important for

Table 2
Combination index values of HDIs and NK92C1

Drug	NK92C1 incubation (h)	Drug conc.	NK92C1 conc.	CI
SAHA	24	1 μM	10^4/ml	0.67
			10^5/ml	0.44
		5 μM	10^4/ml	0.80
			10^5/ml	0.36
	48	1 μM	10^4/ml	0.78
			10^5/ml	0.68
		5 μM	10^4/ml	0.44
			10^5/ml	0.23
NaB	24	1 mM	10^4/ml	0.63
			10^5/ml	0.38
		5 mM	10^4/ml	0.78
			10^5/ml	0.33
	48	1 mM	10^4/ml	0.52
			10^5/ml	0.37
		5 mM	10^4/ml	0.42
			10^5/ml	0.17

CI < 1 and = 1 indicate synergism and additive effect, respectively, $n = 2$.

tumour cell recognition would result in decreased cytolytic activity [25]. In a 4-h ^{51}Cr-release assay without blocking antibodies tumour cells were efficiently killed by PBMCs at an E/T ratio of 40:1 (Fig. 5A–D). Masking any of the NK receptors impaired tumour cell lysis to different extents depending on the tumour cell line. In DAOY cells, masking of NKG2D resulted in a dramatic decrease of cell lysis, whereas masking of DNAM-1 or the NCRs had only minor effects. On the other hand, PC3 cell lysis depended mainly on NCRs and to a lower degree on NKG2D or DNAM-1. Blocking two receptors simultaneously enhanced the protective effect in both cell lines. Blockade of all three receptor types completely abrogated lysis demonstrating that no additional receptors were involved in the recognition and lysis of DAOY or PC3 cells by IL-2-activated PBMCs. As described above, HDI treatment slightly increased tumour cell lysis in the 4-h ^{51}Cr-release assay. However, the lysis of HDI-treated tumour cells was also completely abrogated by the combined masking of NKG2D, DNAM-1 and the NCRs evidencing that HDI treatment did not result in the engagement of alternative activating NK receptors. On the other hand, the protective effect of only one or two blocking antibodies was decreased after HDI treatment of tumour cells. Using IL-2-activated polyclonal NK cells as effector cells we obtained similar results (Fig. 5E and F).

3.5. HDIs increase surface expression of NKG2D ligands on tumour cells

To shed light on the reasons for the improved recognition of tumour cells by NK cells we assessed the influence of HDIs on the expression of activating NK receptor ligands on the tumour cell surface. Basal expression levels of the DNAM-1 ligands PVR and Nectin-2 were high (Fig. 6A), those of the NKG2D ligands ULBPs and MICA were moderate or low in both tumour cell lines. We did not detect the NKG2D ligand MICB. The ligands for the NCRs are not known. Upon SAHA (Fig. 6B) or NaB (data not shown) treatment, all the NK receptor ligands were up-regulated. This was most pronounced for MICA and ULBP2, whose expression density increased more than 4-fold in PC3 cells. Thus, more efficient tumour cell recognition by NK cells due to increased expression of activating NK receptor ligands could be one reason for the enhanced lysis of HDI-treated tumour cells by NK cells.

Since besides apoptosis HDIs induce cell cycle arrest, we have investigated the effects of the cell cycle phase on MICA expression. Fig. 7 demonstrates cell cycle arrest in G_2-M upon tumour cell treatment with 5 μM SAHA. However, MICA was expressed at similar densities throughout the whole cell cycle in both treated and untreated DAOY cells. This excludes HDI-induced cell cycle arrest as an explanation for the increased MICA expression.

3.6. HDIs do not influence the surface expression of death receptors on tumour cells

Finally, we investigated the surface expression of death receptors on tumour cells. PC3 cells expressed Fas and DR5 at high levels and DR4 at a low level. DAOY cells only expressed Fas and DR5 but not DR4 (Fig. 8). HDI treatment slightly decreased the expression of all expressed death receptors. These data argue against the hypothesis that HDIs render tumour cells more susceptible to the death receptor ligands expressed on activated NK cells.

4. Discussion

In this study, we assessed the combined effects of HDIs and activated immune cells from the peripheral blood on cell death induction in the solid tumour-derived cell lines DAOY and PC3. We demonstrate that HDIs and PBMCs synergize to induce tumour cell death in an activating NK receptor-dependent way.

HDIs have been shown to increase the susceptibility for TRAIL in various tumour cell lines (including DAOY and PC3 cells) [11,12,26–29]. This prompted our hypothesis that the effects of HDIs might be partially mediated by immune cells. To test, whether the combination of HDIs with IL-2-activated PBMCs might add to the therapeutic options in difficult to treat tumours, we have chosen

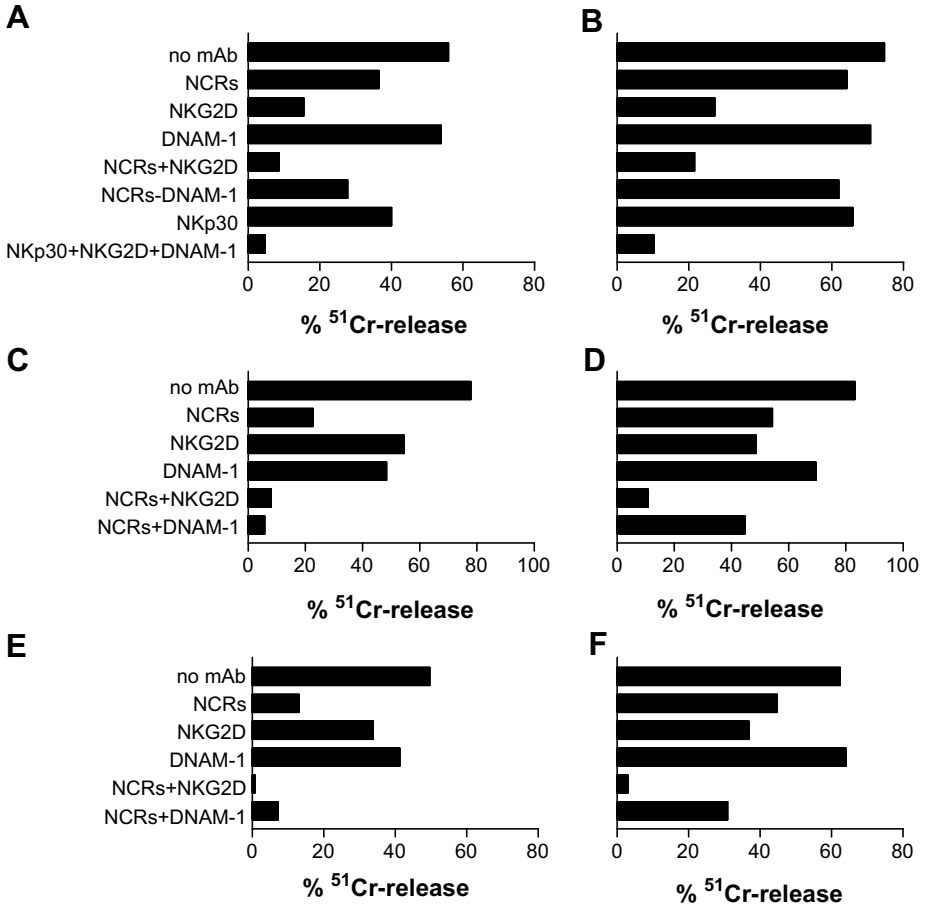

Fig. 5. Involvement of activating NK receptors in tumour cell death induction by IL-2-activated PBMCs and IL-2-activated polyclonal NK cells. ^{51}Cr-labelled tumour cells were used as target cells for IL-2-activated PBMCs at an E/T ratio of 40:1 (A–D) or IL-2-activated polyclonal NK cells at an E/T ratio of 5:1 (E and F) in the presence or absence of masking antibodies against the indicated activating NK receptors. Target cells were untreated DAOY cells (A), DAOY cells pre-treated with 5 μM SAHA (B), untreated PC3 cells (C and E) or PC3 cells pre-treated with 5 μM SAHA (D and F).

two different highly drug-resistant cell lines, DAOY and PC3, for "proof of principle" experiments. We employed HDIs at concentrations typically applied *in vitro* [4,29–31]. These induced between 25% and 50% tumour cell death. For comparison, vincristine was titrated to be similarly effective. IL-2-activated PBMCs also induced tumour cell death in a dose-dependent manner.

Most combinations of the HDIs with IL-2-activated PBMCs showed a very strong synergism according to the criteria of Chou et al. [21]. In contrast, the combination of HDIs and the spindle apparatus-affecting cytostatic vincristine [32] was only moderately synergistic. Our observations are in line with previous reports of an enhanced lysis of HDI-treated tumour cells by IL-2-activated PBMCs [18] or NK cells [33,34]. The sensitizing effect is most likely linked to the deacetylase activity of the HDIs since the cytostatic vincristine had a much lower effect. The sensitization appeared to be reversible, because 48 h after removal of the HDI the synergism was less pronounced than after 24 h. This indicates that not only the direct anti-tumour effects of HDIs are reversible in the absence of the drug [35] but also their immunostimulatory action on tumour cells.

In the 4-h ^{51}Cr-release assay the synergism of HDIs with IL-2-activated PBMCs was much lower than after 24 or 48 h. This suggests that HDIs mainly enhance induction of apoptosis, which is a time-consuming process [36], rather than fast killing mechanisms such as lysis induced by the release of cytotoxic granules [37].

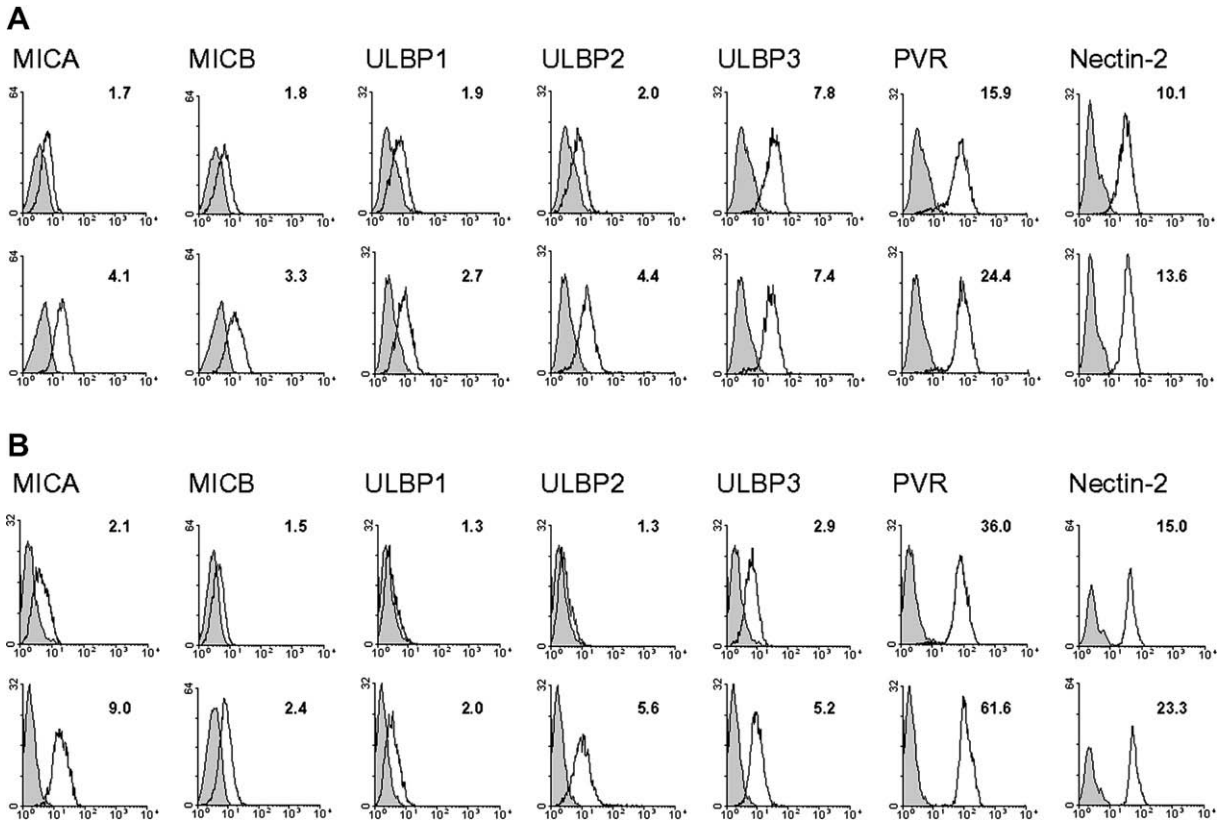

Fig. 6. Surface expression of activating NK receptor ligands on tumour cells. MICA, MICB, ULBP1, ULBP2, ULBP3, PVR and Nectin-2 were examined on DAOY cells (A) and PC3 cells (B). The upper panels show ligand expression in untreated cells, the lower panels show ligand expression in cells treated with 5 μM SAHA. Cells were stained with specific monoclonal antibodies (solid lines) or with isotype control antibodies (filled histograms). Numbers indicate MRFI values.

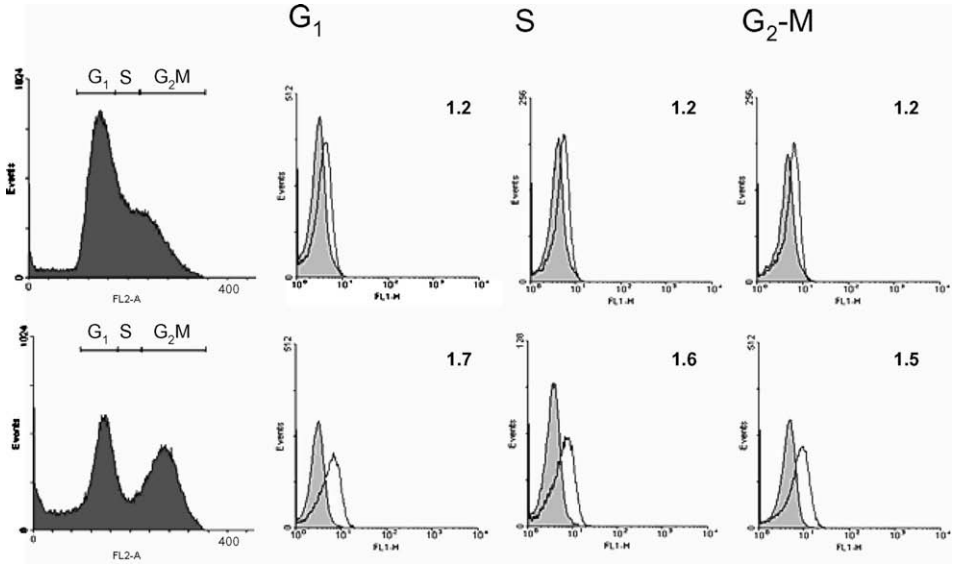

Fig. 7. Cell cycle phases of untreated (upper panel) and SAHA-treated (lower panel) DAOY cells were determined according to PI incorporation into the DNA, and MICA expression in each phase was examined. Cells were stained with specific monoclonal antibodies (solid lines) or with isotype control antibodies (filled histograms). Numbers indicate MRFI values.

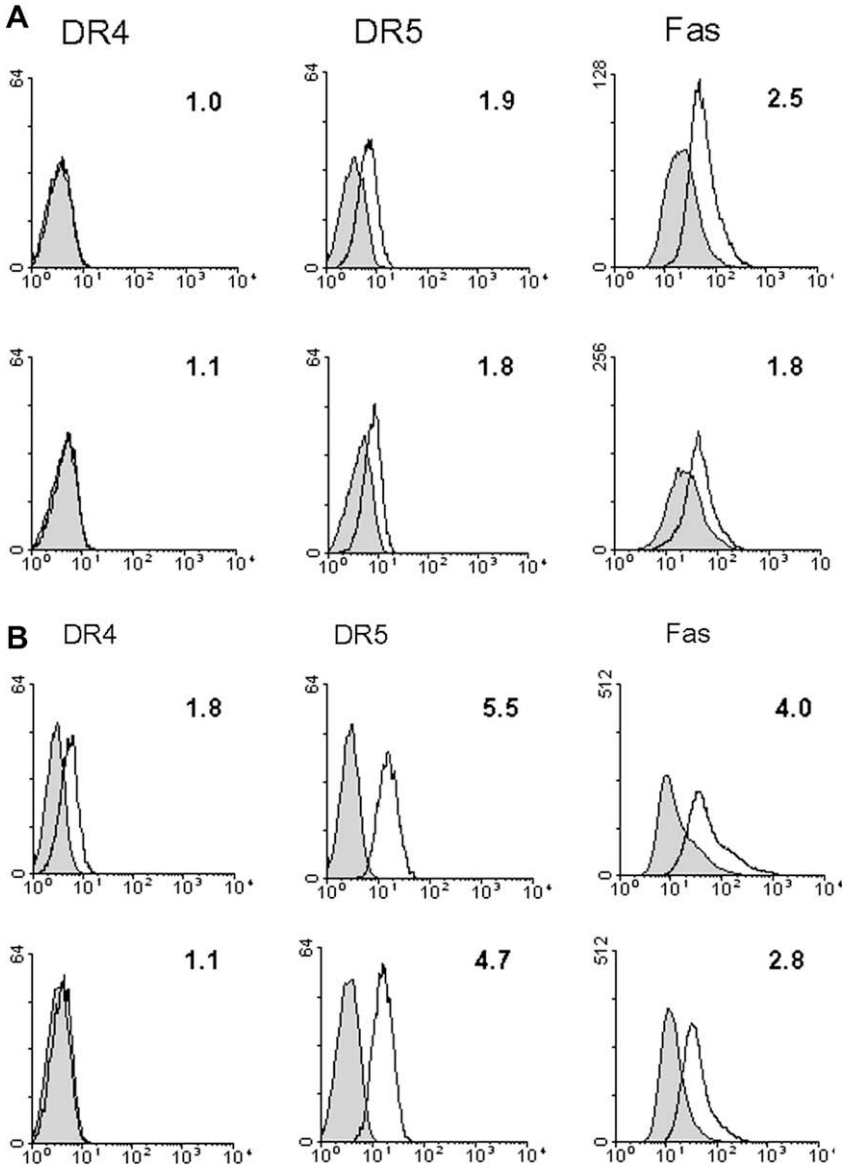

Fig. 8. Surface expression of death receptors on tumour cells. DR4, DR5 and Fas were examined on DAOY cells (A) and PC3 cells (B). The upper panels show expression in untreated cells, the lower panels show expression in cells treated with 5 μM SAHA, respectively. Cells were stained with specific monoclonal antibodies (solid lines) or with isotype control antibodies (filled histograms). Numbers indicate MRFI values.

Following co-incubation of the PBMCs with tumour cells, NK cells but not T cells mobilized the activation marker CD107a [23,24] to their cell surface. This is a strong hint that in our system NK cells are the main effector cells and cytotoxic T cells play at best a marginal role. This fits to the kinetics of the observed cell death after 24 h, because the differentiation of alloreactive $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) takes several days [38,39]. Furthermore, we did not detect the T cell co-stimulatory molecules CD80 and CD86 on HDI-treated tumour cells (data not shown) which would be required to activate naïve $CD8^+$ T cells [40,41]. To investigate a potential sensitizing effect of HDIs on tumour cells for T cell killing the system would need to be modified and the tumour cells exposed to mature tumour-specific CTLs.

The function of NK cells is regulated by the balance between activating and inhibitory signals. In our study, tumour cell lysis by PBMCs was completely abolished when the three major activating NK receptor types, NKG2D, DNAM-1 and NCRs [42], were blocked. This did not change when the tumour cells were pre-treated with HDIs. These findings firstly confirm that NK cells were the crucial effectors in our system. Secondly, they exclude the possibility that induction of alternative NK-activating ligands on tumour cells is important for sensitization. However, HDIs enhanced the redundancy of the involved activating receptors since masking of individual receptors was less effective following HDI exposure.

The most likely explanation for this is the HDI-induced up-regulation of ligands for the activating NK receptors NKG2D and DNAM-1, especially of MICA and ULBP2, on tumour cells after HDI treatment, since the increased ligand density is expected to provide a stronger triggering signal to the NK cells. This is in agreement with previous studies, where VPA and FR901228 were reported to increase MICA and MICB mRNA levels and to up-regulate the expression of these immune-stimulatory molecules on transformed cells. This rendered the tumour cells better targets for NK cell-mediated cytotoxicity [18,34]. Whether the HDI effects were due to increased transcription or to stabilization of mRNA was not examined. A trivial explanation for an increase in MICA expression would be an HDI-induced arrest in a specific cell cycle phase with high basal MICA expression. In fact, HDI treatment arrests the cell cycle in G_1 or G_2-M, dependent on HDI concentration [12,43]. However, we found that MICA was equally expressed in each cell cycle phase. This implies that the observed MICA up-regulation was not due to HDI-related shifts in the distribution of tumour cells in different cycle phases, but represented a real increase of MICA surface expression throughout the cell cycle. Since the NCR ligands are not known yet, no information about changes of their expression could be obtained. Collectively, these findings indicate that facilitation of NK cell triggering contributes to the HDI effect.

It has been reported that HDIs can also sensitize tumour cells for the cytolytic effector mechanisms of immune cells by up-regulating the surface expression of TRAIL, its receptors DR4 and DR5, Fas and FasL [44–47]. In the tumour cells we examined none of these molecules was up-regulated giving no supporting evidence for HDI-sensitization of tumour cells for ligand-based NK kill mechanisms. However, in previous studies, we found HDIs to enhance the sensitivity of DAOY and PC3 cells to TRAIL-induced cytotoxicity although the underlying mechanism could not be disclosed yet [11,12].

If immune cells contribute to the therapeutic effects of HDIs, it would be important to understand, whether these normal cells are directly affected by the drugs. On this point, the published data are controversial. Skov et al. reported that FR901228 inhibited the activation-induced proliferation of normal T cells [48] and Ogbomo et al. observed induction of apoptosis in NK cells and suppression of cytotoxicity in surviving NK cells by SAHA and VPA [49]. This suggests an immunosuppressive effect of HDIs, which was however not confirmed by Tao et al. who reported that the proliferative capacity of T cells from TSA-treated mice was unimpaired [50]. In our experiments, we removed HDIs from the tumour cells before adding IL-2-activated PBMCs and thereby excluded direct drug effects on the immune effector cells.

Our findings indicate that besides their direct tumor-toxic action effector functions of the immune system may contribute to the therapeutic effects of HDIs. Because of possible immune-modulatory effects of HDIs, patients might benefit most from the development of therapeutic regimens that minimize or circumvent immune suppression. For example, the treatment of the tumour with HDIs could be followed by adoptive transfer of *in vitro* activated cytotoxic immune cells as soon as the HDI plasma concentrations have declined.

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