Histone deacetylase inhibition modulates deoxyribonucleotide pools and enhances the antitumor effects of the ribonucleotide reductase inhibitor 3'-C-methyladenosine in leukaemia cells

MARIA MELI[1], MANLIO TOLOMEO[2], MARIO GRIFANTINI[3], ANTONELLO MAI[4], LOREDANA CAPPELLACCI[3], RICCARDO PETRELLI[3], DANTE ROTILI[4], ARIANNA FERRO[1], PHILIPP SAIKO[5], THOMAS SZEKERES[5] and LUISA DUSONCHET[1]

[1]Dipartimento di Scienze per la Promozione della Salute 'G. D'Alessandro'- Sezione di Farmacologia, [2]Centro Interdipartimentale di Ricerca in Oncologia Clinica, Università di Palermo, Policlinico 'P. Giaccone', via del Vespro 129, 90127 Palermo; [3]School of Pharmacy Medicinal Chemistry Unit, University of Camerino, via Sant' Agostino 1, 62032 Camerino; [4]Dipartimento di Chimica e Tecnologie del Farmaco, Università 'La Sapienza', p.le A. Moro 5, 00185 Rome, Italy; [5]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Received October 14, 2010; Accepted December 6, 2010

DOI: 10.3892/ijo.2011.943

Abstract. Histone deacetylase (HDAC) inhibitors are a new class of epigenetic agents that were reported to enhance the cytotoxic effects of classical anticancer drugs through multiple mechanisms. However, which of the possible drug combinations would be the most effective and clinically useful are to be determined. We treated the HL60 and NB4 promyelocytic leukaemia cells with a combination of the ribonucleotide reductase (RR) inhibitor 3'-*C*-methyladenosine (3'-Me-Ado) and several hydroxamic acid-derived HDAC inhibitors, including two recently synthesized molecules, MC1864 and MC1879, and the reference compound trichostatin A (TSA). The results showed significant growth inhibitory and apoptotic synergistic effects with the combinations. Hence, we evaluated the effects of the combinations on cell cycle distribution and on the level of several proteins involved in the apoptotic process (p21, caspase-3, Bcl-2, Bax, AIF). Since HDAC inhibitors increased the G1-S transition block induced by 3'-Me-Ado, an effect on RR activity was hypothesized. Indeed, the HPLC evaluation of intracellular deoxyribonucleotide (dNTP) pools showed that both TSA and MC1864 induced a decrease in dNTPs, even if with a somewhat different pattern, suggesting that RR inhibition contributes to the observed synergism. Furthermore, while TSA was shown to activate the intrinsic apoptotic pathway, MC1864 induced a dose-dependent increase in ROS and AIF levels. Moreover, the treatment with the radical scavenger N-acetylcysteine determined a significant inhibition of MC1864- but not TSA-mediated synergistic effects. Hence, our findings are consistent with a possible role of HDAC inhibitor mediated-ROS induction in RR inhibition and in the potentiation of RR inhibitor-mediated apoptosis.

Correspondence to: Dr Maria Meli, Dipartimento di Scienze per la Promozione della Salute 'G. D'Alessandro'- Sezione di Farmacologia, Policlinico 'P. Giaccone', via del Vespro 129, 90127 Palermo, Italy
E-mail: mmeli@unipa.it

Key words: HDAC inhibitors, RR inhibitors, apoptosis, leukaemia, ROS

Introduction

Epigenetic therapy with histone deacetylase (HDAC) inhibitors represents one of the most intriguing new frontiers among the targeted anticancer therapies. In fact, by favouring the hyperacetylated form of histone proteins, HDAC inhibitors are capable of interfering with the chromatin structure and promoting an euchromatic transcriptionally active state. This leads to the reactivation of a set of genes usually silenced in cancer cells including those involved in the regulation of cell growth, differentiation, and apoptosis (1-4). Indeed, the significant *in vitro* and *in vivo* antitumor activity against a variety of haematological and solid tumor models, together with the relative selectivity toward cancer cells prompted to the rapid testing of a number of these molecules in the clinical setting and the recent approval of vorinostat and romidepsin for the treatment of cutaneous T-cell lymphoma (5-8). However, the precise molecular basis of the anticancer activity of HDAC inhibitors is not completely clear. As a matter of fact, they were reported to exert a series of pleiotropic effects to be ascribed not only to their impact on gene expression, but also to their ability to alter the function of a growing series of non-histone proteins, including transcriptional factors such as p53, signal transduction mediators and molecular chaperones (3,9). Furthermore, the fact that HDAC inhibitors could improve the access of cytotoxic drugs to the target DNA, renders them ideal candidates for combination therapy. Several reports have

shown that these agents synergize *in vitro* and *in vivo* with a number of conventional anticancer drugs such as topoisomerase II inhibitors, taxanes, cisplatin and antimetabolites as well as biologic agents such as imatinib and retinoids (10-13). Their combination with hydroxyurea (HU), a classical ribonucleotide reductase (RR) inhibitor, was also shown to elicit synergistic effects apparently owing to the ability of this latter molecule to counteract the HDAC inhibitors-induced overexpression of the cyclin-dependent kinase inhibitor p21, a putative anti-apoptotic mediator at least in certain experimental models (14). However, HU effectiveness in human leukaemias has been modest due to the well known limits of this drug, namely its relatively low affinity for the RR enzyme, its short half-life and the development of resistance. Since RR has a key role in DNA synthesis and cell growth control, it remains an excellent target for anticancer therapy and new more effective RR inhibitors are currently studied. At present, RR inhibitors are grouped in two main categories depending on their interaction with the R1 or the R2 subunit of the enzyme. In fact, the RR enzyme was shown to possess a tetrameric structure and to be formed by a large α_2-homodimeric R1 subunit which harbors the active and allosteric sites and a small β_2-homodimeric R2 subunit containing an oxigen-linked diferric iron center and a tyrosyl radical that are essential for enzymatic activity (15). We synthesized a new sugar modified nucleoside analog 3'-C-methyadenosine (3'-Me-Ado) with a striking activity as R1-specific RR inhibitor and a significant anti-leukaemic effect *in vitro* (16). Interestingly, we found that its cytotoxic and apoptotic activity could be enhanced by combining it with several hydroxamic acid-derived HDAC inhibitors, including the two recently synthesized molecules, MC1864 and MC1879, and the reference compound trichostatin A (TSA).

Materials and methods

Chemicals and reagents. 3'-C-methyladenosine was synthesized as described (16). The HDAC inhibitors MC1864 and MC1879 were kindly supplied by Professor A. Mai (17). Trichostatin A, hydroxyurea and N-acetyl-L-cysteine (L-NAC) were from Sigma. The caspase inhibitors Z-IETD-FMK (Z-Ile-Glu-Thr-Asp-fluoromethylketone), Z-LEHD-FMK (Z-Leu-Glu (OMe)-His-Asp (OMe)- fluoromethylketone), Z-DEVD-FMK (Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone), Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) and the calpain inhibitor calpeptin were purchased from Alexis Biochemicals (Laufelfingen, Switzerland). The anti-Fas monoclonal antibody (mAb) ZB4 was purchased from Upstate Biotechnology (Lake Placid NY, USA). The mAbs anti-bax, anti-bcl2, anti-p21 were from BD Biosciences (San Jose, CA, USA), while the anti-AIF mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6$) was supplied by Calbiochem (San Diego, CA, USA). All other reagents were analytical grade.

Cell culture and treatment. The human acute promyelocytic cell lines HL60 and NB4 were used in the study. These cell lines do not express P-glycoprotein; furthermore, HL60 cells do not express the fusion gene PML-RAR_α while NB4 ones do. Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated FCS (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 2 mM of L-glutamine (Gibco) at 37˚C in a humidified 5% CO_2 atmosphere.

Cell viability and apoptosis. Cytotoxicity was assessed by the trypan blue dye exclusion test. In brief, cells were seeded at a density of $0.1x10^6$ cells/ml in 24-well plates and, after 24 h incubation in drug-free medium, different concentrations of drugs were added. After 24 or 48 h of exposure, aliquots of cells were harvested, stained with trypan blue and counted on a haemocytometer. All samples were measured in duplicate in at least three independent experiments. For determination of the interactions between drugs the fractional product method of Webb was applied (18). Predicted values (*c*) were calculated according to the equation $c = a \times b/100$ where *a* and *b* indicate cell survival values with single agents. Hence, combination index (CI) was calculated as the ratio between survival observed with the combinations and the predicted values. The equations CI = 1, CI <1 and CI >1 indicate additive effects, synergism and antagonism, respectively.

Apoptosis was evaluated by fluorescence microscopy according to the method of Duke and Cohen (19). After treatment with different concentrations of the drugs for 24-48 h, cells were centrifuged and the pellet was resuspended in 25 μl of a dye mixture containing acridine orange and ethidium bromide. Live and apoptotic cells were identified by fluorescence microscopy. The inhibitory anti-Fas mAb, the caspase inhibitors, L-NAC or calpeptin were added one hour before drug treatment. Alternatively, apoptosis was evaluated by flow cytometry as the percentage of hypodiploid nuclei accumulated in the sub-G_0-G_1 peak after labeling with propidium iodide (20).

Cell cycle analysis. The effects of test compounds on the cell cycle were studied by flow cytometry. Briefly, the cells were washed once in ice-cold PBS and resuspended at $1x10^6$/ml in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 μg/ml in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40 (Sigma). After 30 min of incubation in this solution, the samples were filtered through nylon cloth, 40 μm mesh, and their fluorescence was analyzed as single-parameter frequency histograms by using a FACSort (Becton-Dickinson, Mountain View, CA, USA). A minimum of 10,000 events for each sample was collected in list mode. The distribution of cells in the cell cycle was analyzed with the ModFit LT program (Verity Software House, Inc.).

Evaluation of intracellular dNTP pools by high performance liquid chromatography (HPLC). Logarithmically growing HL60 cells ($0.5x10^6$ per ml) were incubated with 0.1, 0.2 and 0.4 μM TSA or 2, 4 and 8 μM MC1864 for 24 h. Afterwards, $5x10^7$ cells were separated for the extraction of dNTPs according to the method described by Garrett and Santi (21). Cells were centrifuged at 1,800 rpm for 5 min and then resuspended in 100 μl phosphate-buffered saline. In this suspension, cells were lysed by addition of 10 μl of trichloroacetic acid and the mixture was vortexed for 1 min. The lysate was rested on ice for 30 min and then the protein was separated by centrifugation at 15,000 rpm for 10 min in an Eppendorf microcentrifuge. The supernatant was removed and neutralized by adding 1.1 vol

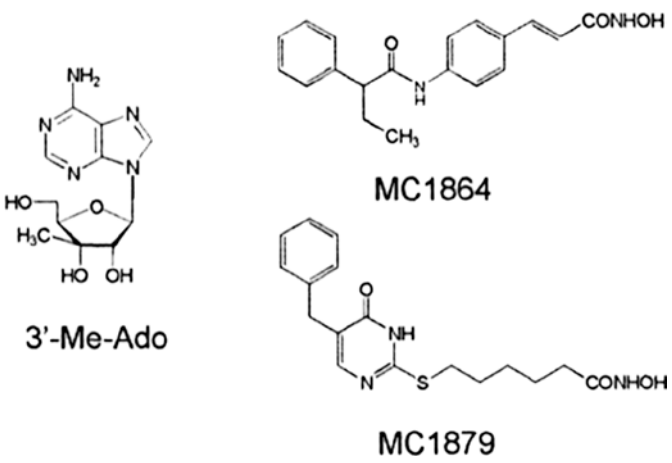


Figure 1. Structure of 3'-Me-Ado and the two hydroxamic acid-derived HDAC inhibitors MC1864 and MC1879.

of Freon containing 0.5 M tri-n-octylamine. Aliquots of 100 μl were periodated by adding 30 μl of 4 M methylamine solution and 10 μl sodium periodate solution (concentration: 100 mg/ml). After incubation at 37˚C for 30 min, the reaction was stopped by adding 5 μl of 1 M rhamnose solution. The extracted dNTPs were measured using a Merck 'La Chrom' high-performance liquid chromatography (HPLC) system (Merck, Darmstadt, Germany) equipped with L-7200 autosampler, L-7100 pump, L-7400 UV detector, and D-7000 interface. Detection time was set at 80 min, with the detector operating on 280 nm for 40 min and then switched to 260 nm for another 40 min. Samples were eluted with a 3.2 mol/l ammonium phosphate buffer, pH 3.4 (pH adjusted by addition of 3.2 mM H_3PO_4), containing 2% acetonitrile using a 4.6x250 mm Partisil 10 SAX analytical column (Whatman Ltd., Kent, UK). Separation was performed at constant ambient temperature with a flow rate of 2 ml/min. The concentration of dNTPs was calculated as percent of total area under the curve for each sample.

Assessment of mitochondrial membrane potential. The mitochondrial membrane potential ($\Delta\Psi$) was measured by flow cytometry after staining the cells with the cationic lipophilic fluorochrome $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide). In brief, treated cells were harvested, washed once in PBS and incubated with the $DiOC_6$ (40 nM) for 15 min at 37˚C. The analysis was carried out by flow cytometry and the percentage of cells exhibiting low levels of $DiOC_6$, reflecting loss of mitochondrial membrane potential, was determined.

Measurement of reactive oxygen species (ROS) production. Levels of ROS were determined by monitoring the oxidation of the probe 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) (Sigma) by flow cytometry. In brief, cells ($4x10^5$) were seeded in 24-well plates and after 24 h were loaded with 100 μM DCFH-DA for 40 min at 37˚C. Thereafter, the cells were exposed to the drugs alone or in combination and as a positive control to H_2O_2 0.5 mM. After a 2-h exposure, the cells were harvested, centrifuged, washed, resuspended in cold serum-free RPMI and the fluorescence was measured by flow cytometry. The increase in ROS level was calculated as the ratio: mean fluorescence intensity of exposed cells/mean fluorescence intensity of unexposed cells x100.

Table I. Cytotoxic effects of the combinations of 3'-Me-Ado and the HDAC inhibitors in HL60 or NB4 cells.

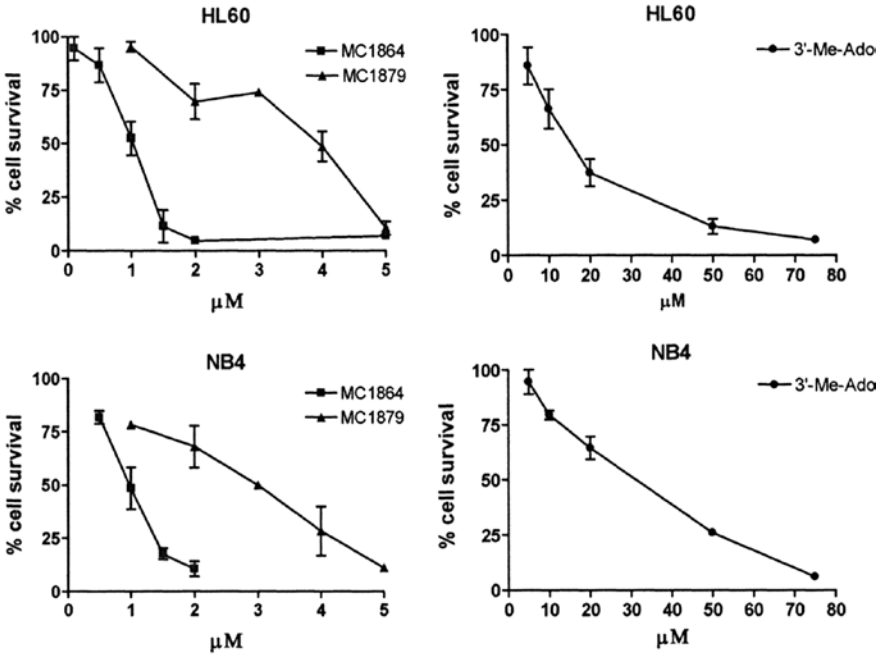
	Treatment	% Cell survival (mean ± SD)	CI
HL60	3'-Me-Ado 20 μM	41±10	-
	MC1864 0.5 μM	66±11	-
	MC1879 4.0 μM	56±2	-
	TSA 0.1 μM	53±14	-
	3'-Me-Ado + MC1864	9±2	0.3
	3'-Me-Ado + MC1879	12±5	0.5
	3'-Me-Ado + TSA	11±6	0.5
NB4	3'-Me-Ado 20 μM	73±1	-
	MC1864 1.0 μM	53±21	-
	MC1879 2.0 μM	49±22	-
	TSA 0.1 μM	64±10	-
	3'-Me-Ado + MC1864	9±4	0.2
	3'-Me-Ado + MC1879	14±8	0.4
	3'-Me-Ado + TSA	17±5	0.4

The cells were exposed to the drugs at the concentrations indicated either alone or in combination for 48 h. Cell survival was evaluated by the trypan blue dye exclusion test. The combination index (CI) was calculated according to the Webb method. A CI ≤0.7 indicates a synergistic effect.

Flow cytometric evaluation of intracellular proteins. About $1x10^6$ cells were washed twice with PBS (Sigma) and resuspended in 100 μl cytofix/cytoperm solution (Becton-Dickinson) at 4˚C. After 20 min the cells were washed twice with BD Perm/Wash™ buffer solution (Becton-Dickinson) and incubated with 20 μl of the specific fluorochrome-conjugated antibody (anti-Bcl-2, -activated caspase 3, -AIF) at 4˚C. After 30 min the cells were washed twice and analysed by flow cytometry. Alternatively, the cells were incubated with 20 μl of a specific unconjugated antibody (anti-p21, anti-bax) at 4˚C for 30 min, washed twice with BD Perm/Wash buffer solution and incubated with 2 μl FITC-conjugated rat anti-mouse IgG1 monoclonal antibody (Becton-Dickinson) at 4˚C. After 30 min the cells were washed twice and analysed by flow cytometry.

Results

HDAC inhibitors and 3'-Me-Ado synergistically inhibit cell growth and trigger apoptosis of human promyelocytic leukaemia cells. We first tested the growth inhibitory and apoptotic effects of 3'-Me-Ado and two new HDAC inhibitors, MC1864 and MC1879 (Fig. 1) in HL60 and NB4 cells. As shown in Fig. 2, all the compounds exerted a dose-dependent growth inhibitory effect at 48 h in both cell lines, with the HDAC inhibitor MC1864 curve showing a somewhat steep progression.



	HL60	NB4
	IC_{50} (μM)	IC_{50} (μM)
MC1864	1.0	1.0
MC1879	4.0	3.0
3'-Me-Ado	15.6	32.1

Figure 2. Growth inhibitory effects of MC1864, MC1879 and 3'-Me-Ado in HL60 and NB4 cells. The cells were exposed for 48 h to increasing concentrations of the compounds and cell survival was evaluated by the trypan blue dye exclusion test. Results are the mean ± SD of at least three separate experiments.

Furthermore, the compounds were able to trigger apoptosis with an AC_{50} of 1.5, 5 and 45 μM for MC1864, MC1879 and 3'-Me-Ado respectively. Based on these results, we selected a concentration of the compounds capable of inducing a moderate inhibition of cell growth (<50%) and only minimally inducing apoptosis (0.5-1 μM for MC1864, 2-4 μM for MC1879 and 20 μM for 3'-Me-Ado) to be used in combination studies. When the cells were exposed to the selected concentration of HDAC inhibitors in combination with 3'-Me-Ado for 48 h, cell growth was inhibited in a synergistic manner (Table I). The synergism was present also when the HDAC inhibitor TSA 0.1 μM was used as a control. On the contrary, when HDAC inhibitors were combined with the RR inhibitor HU, the growth inhibitory effect observed was less than additive (data not shown), suggesting that 3'-Me-Ado and HU act by partially different mechanisms in our cells. Next, we checked whether the combinations were also capable of inducing synergistic apoptotic effects. As shown in Fig. 3, a striking induction of apoptosis was noted in both HL60 and NB4 cells treated with the combinations when compared with single agent, demonstrating a clear synergistic effect. In particular, MC1864 0.5 μM and 3'-Me-Ado 20 μM induced only a 3% and 15% apoptotic rate respectively when tested alone in HL60 cells, whereas the apoptotic effect reached the 76% in the cells treated with the combination. Instead, the combination of HU 0.3 mM with the HDAC inhibitors determined a less marked potentiation of the apoptotic effect (data not shown).

HDAC inhibitors enhance the G1 cell cycle arrest induced by 3'-Me-Ado. To elucidate the mechanisms underlying the observed synergism, we then studied the effect of single compound and combinations on cell cycle distribution in HL60 cells (Table II). The cytofluorimetric results show that all HDAC inhibitors used alone at low concentration did not induce significant effects on cell cycle distribution after 24 or 48 h exposure, while at high fully cytotoxic concentrations they determined an accumulation of the cells in the G_2M phases of the cell cycle after 24 h exposure and a marked increase of the apoptotic sub-G_0G_1 peak after 48 h. On the contrary, 3'-Me-Ado recruited the cells in the G_1 phase in a time-dependent manner, probably as a consequence of its effect on the G_1-S transition. However, this behaviour was more marked in the cells exposed to the RR inhibitor HU at the concentration of 0.5 mM (data not shown). Interestingly, when the cells were exposed to the combination of 3'-Me-Ado and MC1864 or TSA, as a control, we observed a decrease in S phase and an increase in G_1 phase cells particularly after 48 h of exposure, suggesting that HDAC inhibitors potentiate the effect of 3'-Me-Ado on the cell cycle asset. Since previous studies have shown that HDAC inhibitors influence cell cycle checkpoint proteins expression and notably enhance p21 expression in hematopoietic cells (9,22), we evaluated p21 levels in HL60 cells exposed for 24 h to the drugs alone or in combination by flow cytometry. As shown in Fig. 4, HDAC inhibitors at high concentrations increased p21 levels, but this

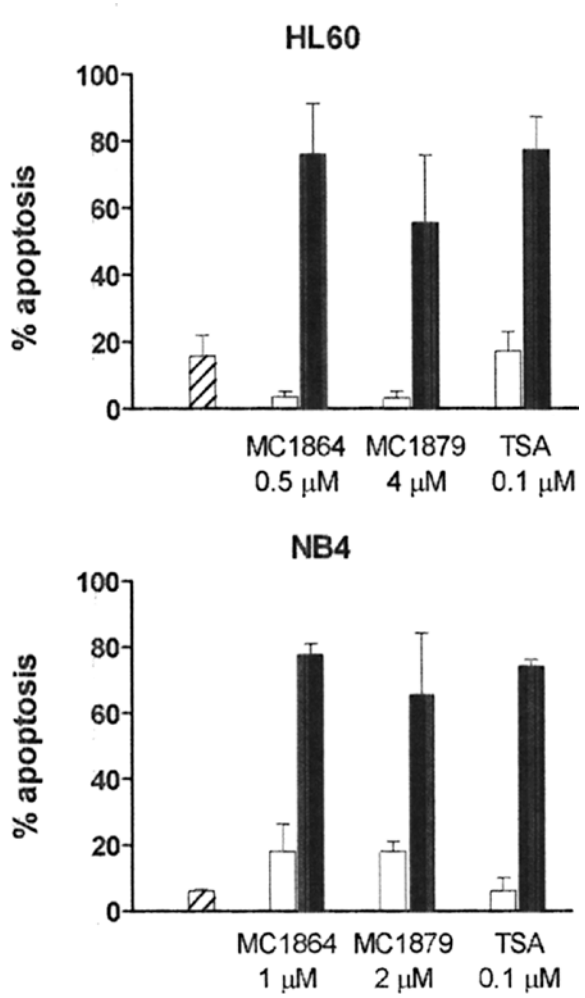


Figure 3. Apoptotic cell death induced by the HDAC inhibitors and 3'-Me-Ado employed alone or in combination in HL60 and NB4 cells. The cells were exposed to the drugs for 48 h and apoptosis was measured by fluorescence microscopy as described in Materials and methods. Dashed column: 3'-Me-Ado alone at the concentration of 20 μM; white column: HDAC inhibitor alone; black column: HDAC inhibitor combined with 3'-Me-Ado. Results are the mean ± SD of at least three separate experiments.

Table II. Cell cycle distribution of HL60 cells exposed to the HDAC inhibitors and/or 3'-Me-Ado.

	G_0G_1	S	G_2M
Control			
24 h	51	39	10
48 h	50	43	7
MC1864 0.5 μM			
24 h	47	40	13
48 h	54	38	8
MC1864 4 μM			
24 h	9	28	63
48 h		NA[a]	
TSA 0.1 μM			
24 h	52	37	11
48 h	48	47	5
TSA 0.5 μM			
24 h	43	13	43
48 h		NA[a]	
3'-Me-Ado 20 μM			
24 h	55	38	5
48 h	68	22	10
3'-Me-Ado 75 μM			
24 h	52	35	12
48 h	55	34	11
MC1864 0.5 μM + 3'-Me-Ado 20 μM			
24 h	54	34	13
48 h	64	30	6
TSA 0.1 μM + 3'-Me-Ado 20 μM			
24 h	59	28	12
48 h	77	19	4

The cells were exposed to the drugs for 24 or 48 h and cell cycle distribution was evaluated by flow cytometry. Values correspond to the percentage of non-apoptotic cells and exclude the sub-G1 population. Values represent the means of three different experiments. [a]NA: not assessable due to the high apoptotic rate.

effect was not evident at the low concentrations used in combinations. Unexpectedly also 0.5 mM HU, but not 3'-Me-Ado, determined an up-regulation of p21 that could contribute to the observed block of the cell cycle at the G_1-S boundary.

HDAC inhibitors depleted deoxyribonucleotide (dNTP) pools in HL60 cells. Next, we checked whether the synergistic effects of HDAC inhibitors may be ascribed to an increase in the RR inhibition mediated by 3'-Me-Ado. Since RR catalyzes the *de novo* conversion of ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates, we measured the intracellular dNTP pools after 24 h exposure to increasing concentrations of MC1864 or TSA in HL60 cells. As shown in Fig. 5, both drugs reduced dNTP pools with a somewhat different profile. MC1864 induced mainly a reduction of dTTP and dATP with a paradoxical increase in dCTP pool at the concentration of 2 μM, while TSA determined a significant depletion of dATP and dGTP and a less marked dCTP depletion at the concentration of 0.4 μM, suggesting that these molecules interfere with dNTP metabolism and possibly interact with the RR enzyme.

Apoptosis induction by the combinations of HDAC inhibitors and 3'-Me-Ado was only partially due to the activation of the intrinsic pathway. In order to clarify the apoptotic pathway involved in the synergism we studied the influence of drugs on the expression of several proteins involved in apoptosis by flow cytometry. The results demonstrated that HDAC inhibitors were able to induce caspase 3 activation and to increase the expression of the mitochondrial protein Bax (less evident with TSA), whereas Bcl-2 expression remained unchanged (Fig. 6). As regards 3'-Me-Ado, we observed also an increase in Bax expression and only a slight caspase 3 activation at the highest concentration of 75 μM. However, when we studied the cells exposed to the combinations, no significant change in the

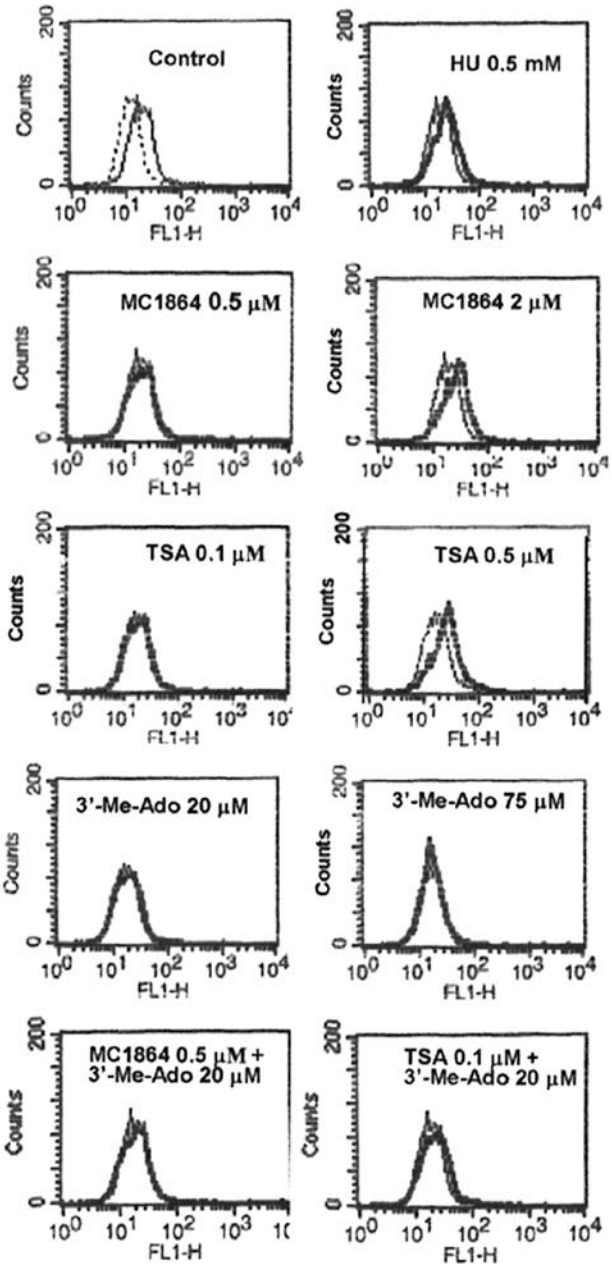


Figure 4. Flow cytometric evaluation of p21 expression in HL60 cells exposed for 24 h to HDAC inhibitors and 3'-Me-Ado alone or in combination. HU was also shown as reference compound. The dotted line indicates the control cells stained with an isotype matched aspecific antibody. The thin and the thick lines represent the untreated control and the treated cells respectively stained with an anti-p21 antibody. The data are representative of three separate experiments.

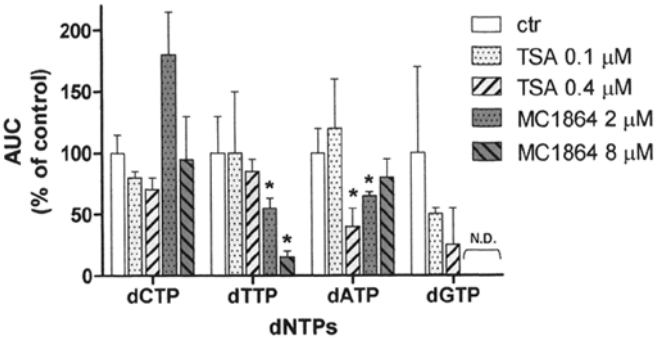


Figure 5. dNTP content of HL60 cells exposed to TSA or MC1864. The cells were exposed to increasing concentrations of the drugs for 24 h and dNTP pools were measured by HPLC. Results are the mean ± SEM of experiments carried out in triplicate and statistical differences between samples are denoted by asterisks. AUC, area under the curve; ND, not determined.

Table III. Effects of HDAC inhibitors and 3'-Me-Ado on the mitochondrial membrane potential ($\Delta\Psi$) in HL60 cells.

	% normal $\Delta\Psi$	% dysrupted $\Delta\Psi$
Control	97	3
MC1864 2 μM	64	36
TSA 0.5 μM	63	37
3'-Me-Ado 75 μM	74	26
3'-Me-Ado + MC1864	80	20
3'-Me-Ado + TSA	90	10

The cells were exposed to the drugs alone or in combination for 24 h and then stained with $DiOC_6$. Fluorescence was evaluated by flow cytometry. Resuls are the means of at least three different experiments.

expression of these apoptotic proteins was noted. To further clear this picture, we then checked whether the molecules either alone or in combination were able to induce a decline in the mitochondrial membrane potential, a typical hallmark of the activation of the intrinsic apoptotic pathway (Table III). The flow cytometric results showed that HDAC inhibitors used alone determined a significant disruption of the mitochondrial membrane potential after 24 h exposure to high but not to low concentrations, whereas 3'-Me-Ado induced only slight decrements. However, the cells exposed for 24 h to the combinations showed only a modest degree of mitochondrial activation (about 20% of cells showing a loss of $\Delta\Psi$), suggesting that drug induced apoptosis was mediated prevalently by a different alternative pathway. Hence, a series of experiments were carried out by employing several caspase inhibitors (caspase 8, 9 and 3 inhibitors) or the anti-Fas moAb ZB4 in order to elucidate the possible involvement of the extrinsic versus the intrinsic apoptotic pathway. The results showed that drug induced apoptosis was always unaffected by the caspase 8 inhibitor or ZB4 (data not shown), excluding an involvement of the extrinsic pathway. As regards the other caspase inhibitors, even if they were found to inhibit the drug-induced caspase 3 activation as evaluated by flow cytometry (data not shown), only the pan-caspase inhibitor Z-VAD reduced apoptosis induced by 3'-Me-Ado or TSA employed alone or in combination; however, it did not influence MC1864-induced apoptosis, suggesting that caspase 3 activation by this latter molecule was not relevant to apoptosis induction (Fig. 7A). Taken together, these data provide support for the notion that apoptosis induced by the combinations does not involve the extrinsic pathway and that the intrinsic pathway was, at least partially, activated, particularly when TSA was used as the HDAC inhibitor.

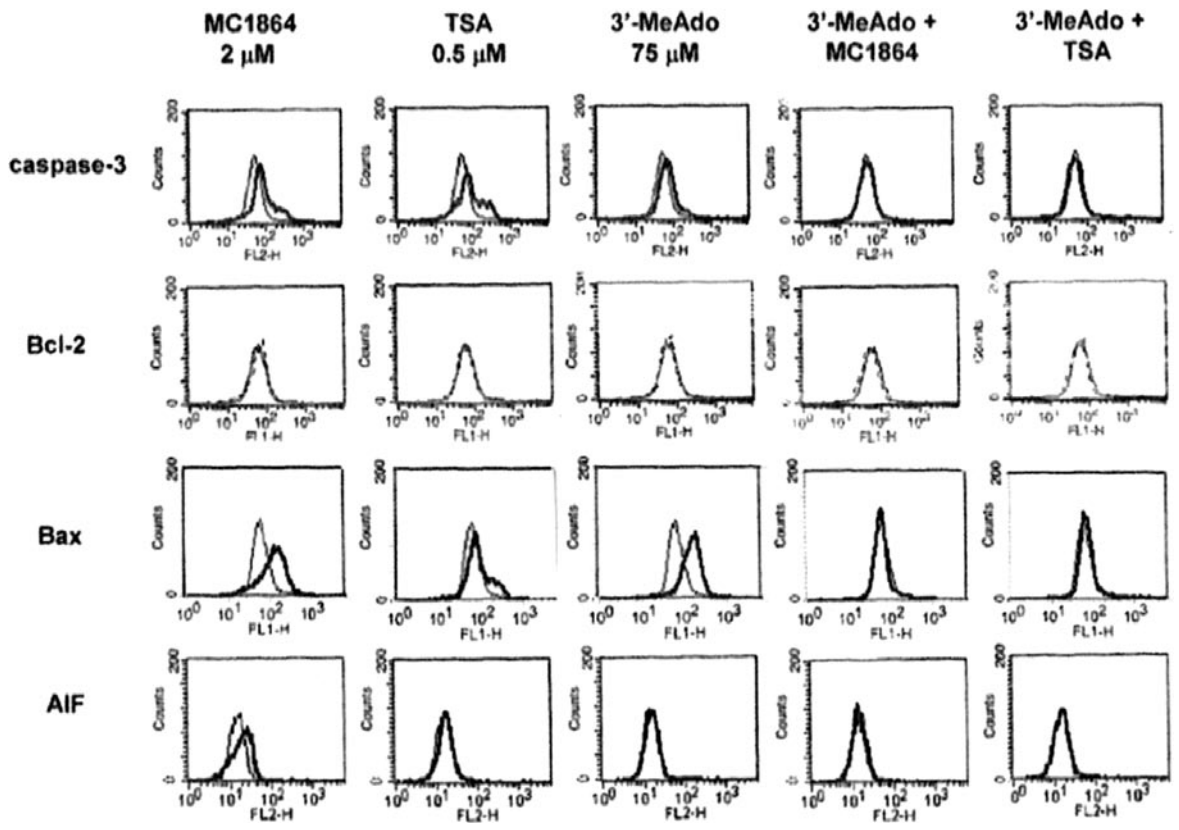


Figure 6. Expression of activated caspase 3, Bcl-2, Bax and AIF in HL60 cells after treatment with HDAC inhibitors and 3'-Me-Ado alone or in combination. The cells were exposed to the drugs at the concentrations indicated for 24 h and protein levels were evaluated by flow cytometry. The thin and the thick lines represent the untreated control and the treated cells respectively stained with the specific monoclonal antibody. The data are representative of three separate experiments.

MC1864-induced apoptosis was associated with ROS generation and AIF release from mitochondria. The induction of ROS has been described to increase mitochondrial membrane permeability, thus promoting apoptosis. We analyzed ROS level in HL60 cells after treatment with HDAC inhibitors and/or 3'-Me-Ado. Fig. 8 shows that MC1864 and TSA induced a dose-dependent increase in ROS production whereas 3'-Me-Ado had no significant effect. Interestingly, 3'-Me-Ado appeared to increase slightly ROS generation by MC1864 0.5 μM and pretreatment with the free radical scavenger L-NAC determined a significant reduction of apoptosis induction by this combination (Fig. 7B). On the contrary, ROS increase by TSA was not affected by the concomitant exposure to 3'-Me-Ado neither L-NAC inhibited TSA-induced apoptosis, demonstrating that TSA and MC1864 both alone or in combination trigger apoptosis through distinct mechanisms in these cells. In particular, these results indicate that ROS production is a key factor in the induction of apoptosis by MC1864, but not TSA, and may contribute to the synergistic apoptotic induction by the combination of MC1864 and 3'-Me-Ado. Since the apoptosis-inducing protein AIF was implicated both in caspase-independent cell death and in antioxidant defence, we checked whether this protein could be induced by MC1864 to explain apoptosis induction by this compound. Indeed, the flow cytometric study indicates that MC1864, but not TSA, was capable of inducing AIF expression in HL60 cells, thus suggesting the activation of a caspase independent pathway by this molecule (Fig. 6). Moreover, AIF increase could be partially reduced by L-NAC pre-treatment (data not shown). AIF is known to translocate from mitochondria to the nucleus where it can induce DNA fragmentation, a process that can be triggered by cellular proteases such as calpain (23). We tested whether the exposure to the calpain inhibitor calpeptin could modify MC1864 induced apoptosis in HL60 cells (Fig. 7C). Unexpectedly, calpeptin paradoxically increased MC1864 induced apoptosis while it inhibited the synergistic effects of the combination with 3'-Me-Ado, suggesting that an unconventional pathway may be involved in the process.

Discussion

HDAC inhibitors are a new class of epigenetic anticancer agents endowed with the ability to significantly target cell protein expression. Even if clinical results obtained so far with these compounds employed alone have been modest, it has been recognized that they could have the potential for being combined with other anticancer drugs in order to enhance their effects especially for the treatment of haematological malignancies (24). In vitro synergistic effects were reported by several authors, employing both conventional and biological agents and, even if the mechanisms underlying the observed synergisms were not entirely cleared, HDAC inhibitors seem mostly to promote drug-induced cell death by

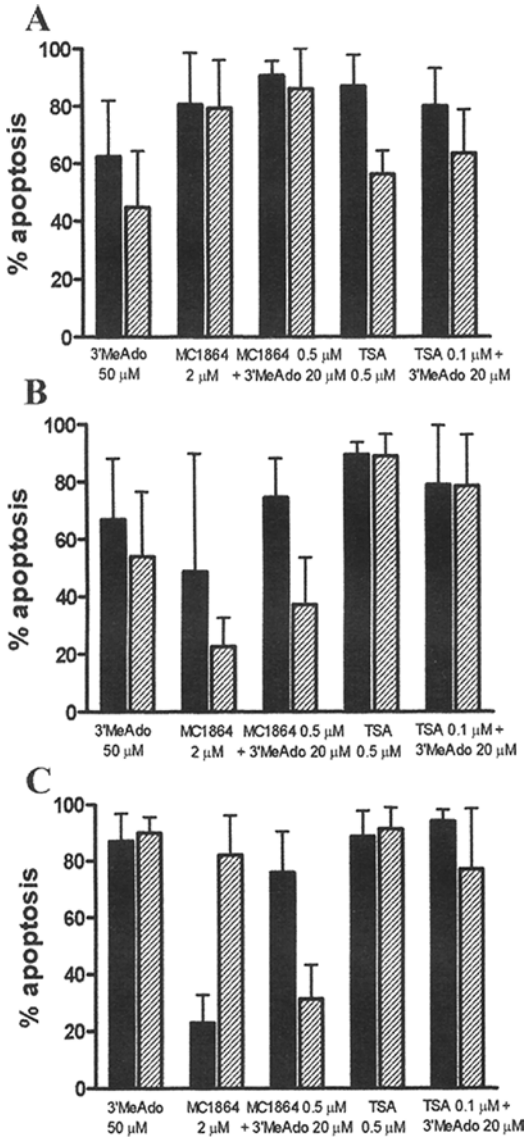


Figure 7. Influence of the pan-caspase inhibitor Z-VAD (A), the radical scavanger L-NAC (B) or the calpain inhibitor calpeptin (C) on apoptosis induced by the HDAC inhibitors and 3'-Me-Ado alone or in combination in HL60 cells. The cells were exposed to Z-VAD, L-NAC or calpeptin for 2 h and then treated with MC1864, TSA, 3'-Me-Ado or the combinations for 48 h. Apoptosis was evaluated by fluorescence microscopy. The black columns show the percentages of apoptosis after treatment with the indicated drugs; the dashed columns show apoptosis after the same treatments in combination with Z-VAD 50 μM (A), L-NAC 10 mM (B) or calpeptin 20 μM (C). Results are the mean ± SD of at least three separate experiments.

modifying the levels of a series of proteins involved in the activation or the inhibition of the apoptotic process (9). However, in some instances they proved to interfere directly with the specific drug target as in the case of etoposide whose cytotoxicity could be enhanced by the HDAC inhibitor-mediated increases in topoisomerase II levels (25).

In this study, we demonstrate that an interesting therapeutic option could be the combination of HDAC inhibitors with an RR inhibitor for the therapy of leukaemia. Even if synergistic effects employing other RR inhibitors, namely hydroxyurea and gemcitabine, have been already reported, our data differ significantly from those of others (11,14). First, we used a newly synthesized, entirely mechanism-based designed, RR inhibitor, 3'-*C*-methyladenosine, a sugar-modified adenosine derivative that competes with the substrate on the R1 subunit of the enzyme (16). Second, interestingly we show that the compounds interact at different levels: on one hand, a significant increase in the growth inhibitory effect of the combinations was observed in both HL60 and NB4 cells when compared with the drugs employed alone; on the other, a more striking potentiation of the apoptotic effect was noted. We believe that different mechanisms could account for these observations. Indeed, although HDAC inhibitors employed alone caused a block of HL60 cells in the G_2 phase of the cell cycle, the cells treated with the combinations appear to be recruited in the G_1 phase, a typical behaviour of RR inhibitors. Thus, we hypothesized that the HDAC inhibitors increased the antiproliferative effect of 3'-Me-Ado. This was not due to an increase in p21 expression, whose levels were raised only when the cells were exposed to the HDAC inhibitors at the highest concentrations. Instead, MC1864 and TSA were shown to reduce variably the intracellular dNTP levels at concentrations near to those employed in combination. The paradoxical increase in dCTP pool induced by MC1864 could be explained by the activation of rescue mechanisms as reported also with HU (26). Thus, we speculate that HDAC inhibitors could inhibit on their own the RR enzyme possibly through a ROS-mediated inactivation of the R2 subunit of the enzyme. Indeed, it is well known that the diiron-tyrosyl radical center of R2 protein is very labile and sensitive to the oxidative milieu (27). In this way, a double inhibition, both R1- and R2-mediated, could explain the synergistic effect of the combination with 3'-Me-Ado. This hypothesis would be supported also by the absence of synergism between HDAC inhibitors and the R2-inhibitor HU in our model, because both compounds would share the same target resulting in additive or antagonistic effects.

Furthermore, some hydroxamic acid-containing RR inhibitors, which are capable of acting specifically on the R2 subunit, have been described (28,29). These compounds were reported to decrease both purine and pyrimidine dNTP levels as found also by us. However, an effect of HDAC inhibitors on other steps of dNTP metabolism cannot be excluded.

As regards the apoptotic effects, HDAC inhibitors are known to produce a major influence on a series of pro-apoptotic proteins and in particular TSA was reported to activate a p53-mediated mitochondrial pathway that involves several components of the Bcl-2 family proteins such as Bim/Bid and caspase 9 activation (11,30,31). However, the pathways involved appeared largely model-dependent (30). In our promyelocytic cells all the hydroxamic acid derivatives and 3'-Me-Ado significantly potentiate the pro-apoptotic effects of each other, but through different molecule-dependent mechanisms. Whereas TSA effects involve a caspase-dependent mitochondrial pathway and are inhibited by the specific caspase inhibitor Z-VAD, MC1864 activates a caspase-independent ROS- mediated process. ROS were repeatedly reported to mediate some effects of HDAC inhibitors and although the basis of ROS generation by these molecules were not clearly elucidated, it appears to be a class-specific effect (32-34). In our HL60 cells HDAC inhibitor-mediated ROS generation appeared to be a general and early phenomenon, being evident as soon as 2 h from drug exposure; yet, pretreatment with the antioxidant L-NAC significantly reduced drug-induced apoptosis only

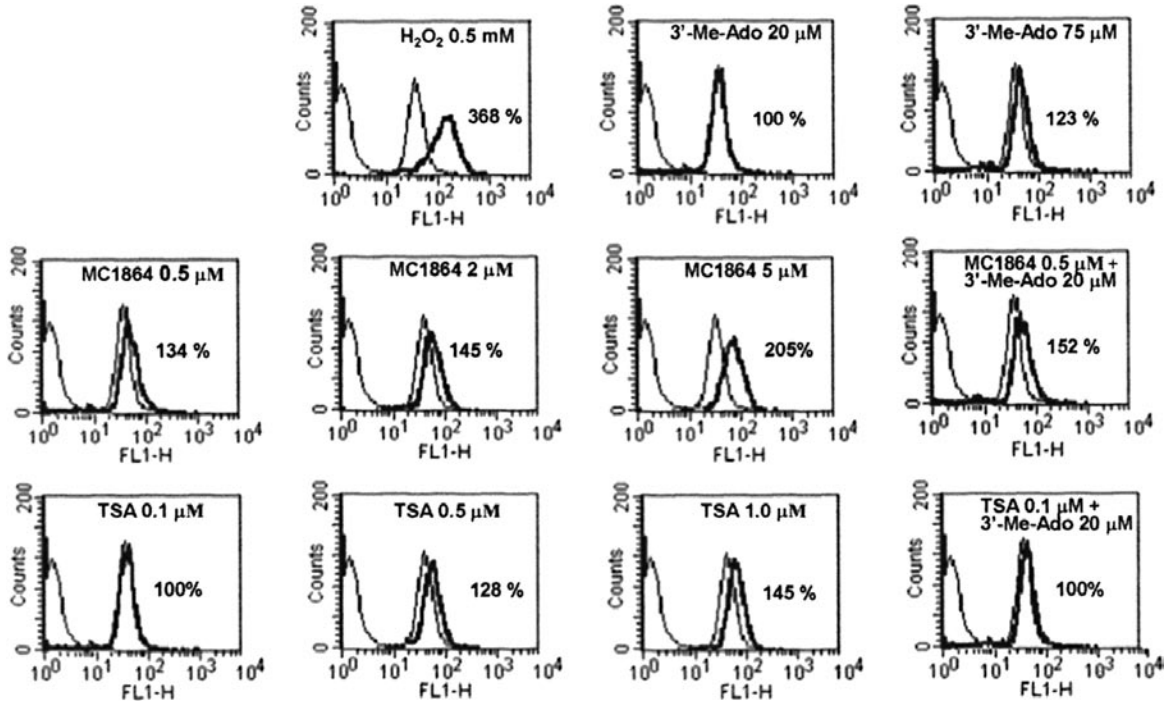


Figure 8. Flow cytometric evaluation of ROS production by HL60 cells exposed to the HDAC inhibitors alone or combined with 3'-Me-Ado. The cells were loaded with the probe DCFH-DA for 40 min, exposed to the drugs for 2 h and examined by flow cytometry. The thin and the thick lines indicate the untreated control sample and the treated one, respectively; the curves on the left represent the blank sample. As a positive control, the cells were exposed to H_2O_2 0.5 mM. Reported values are the mean of at least three separate experiments.

when the new inhibitor MC1864 was employed. This latter molecule, thus, appears to involve a somewhat different molecular pathway that includes also an increase in Bax expression and AIF release from mitochondria. AIF is a well known mediator of caspase-independent apoptosis but it is also a component of cellular antioxidant defenses. It is a protein normally anchored to the mitochondrial inner membrane, but upon apoptosis induction it is cleaved by calpain or other proteases and released in the cytoplasm. Moreover, enhanced formation of ROS was recently reported to be a prerequisite for AIF release in certain models (35). Indeed, MC1864-induced AIF release was partially inhibited by L-NAC but not by the calpain inhibitor calpeptin (data not shown), suggesting that this event can be a consequence of ROS increase, whereas it appears independent from the classical calpain-activated pathway. The paradoxical increase of MC1864-dependent apoptosis by calpeptin could be due to the possible anti-apoptotic effects of calpain that have been attributed to the inactivating cleavage of some apoptotic mediators such as caspase 9 (36,37).

Interestingly, the combination of MC1864 and 3'-Me-Ado seems to depict a partially different picture, possibly due to the much lower concentrations used. We noted that the majority of the molecular alterations observed at high concentrations were not evident in the cells exposed to the combination, including the modifications in Bax and AIF expression. Instead, the facts that, i) when combined, 3'-Me-Ado increased ROS generation by MC1864 and ii) L-NAC significantly reduced the apoptotic effects of the combination, suggest that a ROS-mediated effect was the basis of the synergism. However, unexpectedly also calpeptin showed a significant inhibitory effect on apoptosis induction by the combination: in the absence of a significant release of AIF by the combination and considering that the inhibitory effect induced by the pretreatment with both L-NAC and calpeptin was not higher than that observed with each inhibitor alone (data not shown), we speculate that ROS generation and calpain activation can be related events, even if AIF-independent, in this model.

In conclusion, in this report we demonstrate for the first time that HDAC inhibitors influence dNTP metabolism probably by inhibiting the RR enzyme. Therefore, the combination of HDAC and RR inhibitors may represent a new therapeutic tool to be explored in the clinics for the treatment of leukaemia patients due to the highly synergistic effects achievable. Moreover, our findings support the idea that HDAC inhibitor mediated-ROS induction could have a key role in RR inhibition and in the potentiation of RR inhibitor-mediated apoptosis.

Acknowledgement

This study was supported by the Italian MIUR- PRIN 2006 and Fondazione Roma.

References

1. Hellebrekers DMEI, Griffioen AW and van Engeland M: Dual targeting of epigenetic therapy in cancer. Biochim Biophys Acta 1775: 76-91, 2007.
2. Hess-Stumpp H: Histone deacetylase inhibitors and cancer: from cell biology to the clinic. Eur J Cell Biol 84: 109-121, 2005.
3. McLaughlin F and La Thangue NB: Histone deacetylase inhibitors open new doors in cancer therapy. Biochem Pharmacol 68: 1139-1144, 2004.
4. Mehnert JM and Kelly WK: Histone deacetylase inhibitors: Biology and mechanism of action. Cancer J 13: 23-29, 2007.
5. Batty N, Malouf GG and Issa JPJ: Histone deacetylase inhibitors as antineoplastic agents. Cancer Lett 280: 192-200, 2009.

6. Campas-Moya C: Romidepsin for the treatment of cutaneous T-cell lymphoma. Drugs Today 45: 787-795, 2009.
7. Glaser KB: HDAC inhibitors: clinical update and mechanism-based potential. Biochem Pharmacol 74: 659-671, 2007.
8. Piekarz RL, Sackett DL and Bates SE: Histone deacetylase inhibitors and demethylating agents: Clinical development of histone deacetylase inhibitors in cancer therapy. Cancer J 13: 30-39, 2007.
9. Carew J, Giles FJ and Nawrocki ST: Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. Cancer Lett 269: 7-17, 2008.
10. Bevins RL and Zimmer SG: It's about time: scheduling alters effect of histone deacetylase inhibitors on camptothecin-treated cells. Cancer Res 65: 6957-6966, 2005.
11. Donadelli M, Costanzo C, Begelli S, *et al*: Synergistic inhibition of pancreatic adenocarcinoma cell growth by trichostatin A and gemcitabine. Biochim Biophys Acta 1773: 1095-1106, 2007.
12. Fuino L, Bali P, Wittmann S, *et al*: Histone deacetylase inhibitor LAQ824 down-regulates Her-2 and sensitizes human breast cancer cells to trastuzumab, taxotere, gemcitabine, and epothilone B. Mol Cancer Ther 2: 971-984, 2003.
13. Maggio SC, Rosato RR, Kramer LB, *et al*: The histone deacetylase inhibitor MS-275 interacts synergistically with fludarabine to induce apoptosis in human leukaemia cells. Cancer Res 64: 2590-2600, 2004.
14. Kramer OK, Knauer SK, Zimmermann D, Stauber RH and Heinzel T: Histone deacetylase inhibitors and hydroxyurea modulate the cell cycle and cooperatively induce apoptosis. Oncogene 27: 732-740, 2008.
15. Shao J, Zhou B, Chu B and Yen Y: Ribonucleotide reductase inhibitors and future drug design. Curr Cancer Drug Targets 6: 409-431, 2006.
16. Franchetti P, Cappellacci L, Pasqualini M, *et al*: Antitumor activity of C-methyl-β-D-ribofuranosyladenine nucleoside ribonucleotide reductase inhibitors. J Med Chem 48: 4983-4989, 2005.
17. Savarino A, Mai A, Norelli S, *et al*: 'Shock and kill' effects of class I-selective histone deacetylase inhibitors in combination with the glutathione synthesis inhibitor buthionine sulfoximine in cell line models for HIV-1 quiescence. Retrovirology 6: 52, 2009.
18. Webb JL: Effect of more than one inhibitor. In: Enzymes and Metabolic Inhibitors. Academic Press, New York, pp66-79, 1966.
19. Duke RC and Cohen JJ: Morphological and biochemical assays of apoptosis. In: Current Protocols in Immunology. Coligan JE and Kruisbeak AM (eds.), John Wiley & Sons, New York, p 3.17.1., 1992.
20. Darzynkiewicz Z, Bruno S, Del Bino G, *et al*: Features of apoptotic cells measured by flow cytometry. Cytometry 13: 795-798, 1992.
21. Garrett C and Santi DV: A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleoside triphosphates in cell extracts. Anal Biochem 99: 268-273, 1979.
22. Rosato RR, Almenara JA and Grant S: The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukaemia cells through a process regulated by generation of reactive oxygen species and induction of p21 CIP1/WAF1. Cancer Res 63: 3637-3645, 2003.
23. Lorenzo HK and Susin SA: Therapeutic potential of AIF-mediated caspase-independent programmed cell death. Drug Res Updat 10: 235-255, 2007.
24. Stimson L, Wood V, Khan O, Fotheringham S and La Thangue NB: HDAC inhibitor-based therapies and haematological malignancy. Ann Oncol 20: 1293-1302, 2009.
25. Kurz EU, Wilson SE and Leader KB: The histone deacetylase inhibitor sodium butyrate induces DNA topoisomerase II α expression and confers hypersensitivity to etoposide in human leukemic cell lines. Mol Cancer Ther 1: 121-131, 2001.
26. Bianchi V, Pontis E and Reichard P: Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. J Biol Chem 261: 16037-16042, 1986.
27. Zhu L, Zhou B, Chen X, Jiang H, Shao J and Yen Y: Inhibitory mechanisms of heterocyclic carboxaldehyde thiosemicarbazones for two forms of human ribonucleotide reductase. Biochem Pharmacol 78: 1178-1185, 2009.
28. Elford HL and van't Riet B: Inhibition of nucleoside diphosphate reductase by hydroxybenzohydroxamic acid derivatives. Pharmac Ther 29: 239-254, 1985.
29. Elford HL, Wampler GL and van't Riet B: New ribonucleotide reductase inhibitors with antineoplastic activity. Cancer Res 39: 844-851, 1979.
30. Henderson C and Brancolini C: Apoptotic pathways activated by histone deacetylase inhibitors: implication for the drug-resistant phenotype. Drug Res Updat 6: 247-256, 2003.
31. Henderson C, Mizzau M, Paroni G, Maestro R, Schneider C and Brancolini C: Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). J Biol Chem 278: 12579-12589, 2003.
32. Bouzar AB, Boxus M, Defoiche J, *et al*: Valproate synergizes with purine nucleoside analogs to induce apoptosis of B-chronic lymphocytic leukaemia cells. Br J Heamatol 144: 41-52, 2008.
33. Mitsiades N, Mitsiades CS, Richardson PG, *et al*: Molecular sequelae of histone deacetylase inhibition in human malignant B cells. Blood 101: 4055-4062, 2003.
34. Ruefli AA, Ausserlechner MJ, Bernhard D, *et al*: The histone deacetylase inhibitor and chemotherapeutic agent suberoylanilide hydroxamic acid (SAHA) induces a cell-death pathway characterized by cleavage of Bid and production of reactive oxygen species. Proc Natl Acad Sci 98: 10833-10838, 2001.
35. Norberg E, Gogvadze V, Vakifahmetoglu H, Orrenius S and Zhivotovsky B: Oxidative modification sensitizes mitochondrial apoptosis-inducing factor to calpain-mediated processing. Free Radic Biol Med 48: 791-797, 2010.
36. Chua BT, Guo K and Li P: Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. J Biol Chem 275: 5131-5135, 2000.
37. Goll DE, Thompson VF, Li H, Wei W and Cong J: The calpain system. Physiol Rev 83: 731-801, 2003.
38. Carraway HE and Gore SD: Addition of histone deacetylase inhibitors in combination therapy. J Clin Oncol 25: 1955-1956, 2007.