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Review

Sensitization of tumor cells by targeting histone deacetylases

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ABSTRACT

Epigenetic mechanisms may contribute to drug resistance by interfering with tumor growth regulatory pathways and pro-apoptotic programs. Since gene expression is regulated by acetylation status of histones, a large variety of histone deacetylase (HDAC) inhibitors have been studied as antitumor agents. On the basis of their pro-apoptotic activity, HDAC inhibitors have been combined with conventional antitumor agents or novel target-specific agents to increase susceptibility to apoptosis and drug sensitivity of cancer cells. Several combination strategies including HDAC inhibitors have been explored in preclinical studies. Promising therapeutic effects have been reported in combination with DNA damaging agents, taxanes, targeted agents, death receptor agonists and hormonal therapies. Some histone deacetylases, such as HDAC6, can also modulate the function of non-histone proteins involved in critical regulatory processes which may be relevant as therapeutic targets. Given the pleiotropic effects of most of the available inhibitors, the mechanisms of the sensitization are not completely elucidated. A better understanding of the involved mechanisms will provide a rational basis to improve the therapeutic outcome of the available antitumor agents.

Contents

1. Introduction

The limited efficacy of antitumor therapy reflects the resistance of tumor cells which is a common phenomenon in solid tumors, most evident in the advanced metastatic disease. A number of mechanisms have been implicated in the development of drug resistance, including gene mutations, genome alterations and epigenetic changes [1]. These changes may affect the expression of proteins that influence cellular pharmacokinetics, drug metabolism or cellular response to drug treatment. In addition, evidence has been accumulated to support the involvement of tumor microenvironment in mediating resistance of solid tumors [2,3]. The microenvironment of solid tumors and metastases may influence the response to treatments and may activate epigenetic mechanisms that can lead to transient drug resistance [4]. Epigenetic changes have been implicated in tumor development and progression, because these changes may result in activation of oncogenes and inhibition of the function of tumor suppressors or pro-apoptotic genes [5,6]. Histone acetylation, which is the result of the balance between the activity of histone deacetylases (HDAC) and histone acetyl transferases, is recognized to play an important role in the regulation of gene expression [7,8]. Acetylation of histones promotes a relaxed chromatin structure, leading to transcriptional activation; in contrast, HDAC enzymes can act as transcriptional repressors by deacetylating histones, thereby

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promoting chromatin condensation. HDAC enzymes may be deregulated in many tumors and may be implicated in silencing of growth regulatory pathways and of pro-apoptotic programs [9]. These features and, in particular, the failure to undergo apoptosis in response to antitumor agents may be important determinants of drug resistance [10]. In addition to histones, HDAC can modulate the function of other proteins involved in critical regulatory processes, including proliferation and angiogenesis [9].

The implication of HDAC in oncogenic pathways has generated a great interest for these enzymes as potential therapeutic targets. Thus, although the HDAC inhibitors have been developed as promising antitumor agents for their potential ability to reverse repressive epigenetic alterations associated with drug resistance, their pleiotropic effects could be better exploited in rationally designed combinations, including both conventional cytotoxic agents and molecularly targeted therapy. In this regard, the ability of HDAC inhibitors to induce tumor cell apoptosis has suggested therapeutic potential of these agents in combination strategies aimed at enhancing cell death mediated by pro-apoptotic agents.

2. Sensitization to DNA damaging agents

Acetylation of histones results in chromatin relaxation, thus increasing DNA accessibility for binding of DNA damaging agents. The open chromatin status may expose DNA to the action of exogenous and endogenous DNA damaging agents, including the reactive oxygen species. Chromatin structure is known to influence radiation-induced cell killing [11,12]. The chemosensitization effects of HDAC inhibitors have been observed in a variety of tumor types treated with different DNA damaging agents. Inhibition of HDAC by trichostatin A or suberoylanilide hydroxamic acid (SAHA, Vorinostat), two pan-HDAC inhibitors, increases the cytotoxicity of several DNA damaging agents, characterized by different mechanism of action, including topoisomerase inhibitors, cisplatin and ionizing radiation [13–15]. Moreover, in addition to chromatin modulation resulting in increased DNA damage, HDAC inhibitors may block DNA repair, because they prolong the expression of markers of DNA-damage response (e.g., γH2AX) [13]. Indeed, a large number of proteins implicated in cellular response to DNA damage (e.g., p53 and Ku70) are known to be targets of lysine acetylation [8]. Not only p53, but also proteins of its interaction network are regulated by acetylation [8]. Thus, sensitization to DNA damaging agents is not solely dependent on alteration of chromatin structure, but it also involves the DNA damage response.

A recent study documents that inhibition of HDAC6 by tubacin enhances the cytotoxic effects of DNA damaging agents in tumor cells, an effect not observed in normal cells (i.e., human fibroblasts) [16]. Although the basis of this selectivity of HDAC inhibition is not clear yet, it is likely that the different response reflects an increased ability to repair DNA damage by normal cells. HDAC6 inhibition by tubacin enhanced the apoptotic response to the DNA topoisomerase II inhibitor, etoposide, an effect associated with increased DNA damage. Tubacin itself induced DNA damage as indicated by accumulation of the early sensitive marker of DNA double-strand breaks γH2AX, and by activation of DNA damage checkpoint kinases [16]. The activity of tubacin is relevant to better understand the cellular basis of interaction with cytotoxic agents. Indeed, this hydroxamic acid-based compound is a selective inhibitor of the cytoplasmic HDAC6, because it induces tubulin acetylation without affecting histone acetylation [17]. The use of tubacin as a probe allowed to highlight the cellular functions of HDAC6 [9]. The effects of tubacin in combination with DNA damaging agents are reminiscent of sensitization of ovarian carcinoma cells to the atypical retinoid ST1926 by a novel HDAC inhibitor (ST2782), characterized by preferential inhibition of HDAC6 and ability to induce p53 hyperacetylation [18]. Previous studies from our laboratory support that DNA damage is a primary event in the mechanism of action of the adamantyl retinoids including ST1926 [19]. As observed for ionizing radiation [20], the sensitization to ST1926-induced apoptosis by the HDAC inhibitor was associated with prolonged expression of DNA damage markers (i.e., phosphorylation of H2AX and p53) and enhanced activation of checkpoint kinases.

A comparison of drug effects in ovarian carcinoma cells with wild-type p53 or mutant p53 supports that the functional p53 may confer susceptibility to a rapid onset of HDAC inhibitor-induced apoptosis [18]. The implication of p53 in response to HDAC inhibitors has been observed in a recent study showing that MS-275 (Entinostat) enhanced apoptosis induced by DNA damaging agents (topoisomerase II inhibitors) in medulloblastoma cells [21]. Such a sensitization reflects enhanced p53-dependent activation of Bax. Indeed, MS-275 causes acetylation of the Ku70 protein resulting in Bax release and activation of the mitochondrial pathway of apoptosis. p53 acetylation and phosphorylation following DNA damage may be enhanced by HDAC inhibitors resulting in cooperation to promote apoptosis in wild-type p53 tumor cells [18]. A comparison of isogenic tumor cells with different levels of p53 supports the influence of p53 expression in radiosensitization by trichostatin A [15]. Indeed, p53 appears to be implicated in regulation of susceptibility to apoptotic cell death in response to HDAC inhibitors [18,22]. Thus, although under some circumstances tumor cells with functional p53 may be more responsive to HDAC inhibitors, these agents may also induce p53-independent apoptosis likely as consequence of their pleiotropic effects on regulatory pathways [23].

Various HDAC inhibitors with different HDAC inhibition profile have been reported to induce DNA damage [16,24]. Since there is no evidence of direct DNA damage by HDAC inhibitors [25], it is conceivable that DNA damage after exposure to such compounds is an indirect event likely reflecting a widespread modulation of stress response [23]. In particular, various HDAC inhibitors have been reported to be able to induce oxidative stress with accumulation of reactive oxygen species resulting in oxidative DNA damage [12,25]. This interpretation implicating oxidative stress is supported by the observation that genotoxic stress may be induced by inhibition of the cytoplasmic HDAC6 [16]. HDAC6-triggered damage is expected to cooperate with other genotoxic stress induced by cytotoxic agents leading to synergistic induction of cell death [18].

In addition, HDAC inhibitors may produce genomic instability, mediated by different mechanisms. SAHA was reported to cause alterations in DNA replication resulting in DNA damage with activation of histone γH2AX [26]. This event was unrelated to apoptosis, because it occurred within 4 h of exposure. An early genotoxic stress was also observed in tumor cells treated with HDAC inhibitors of a novel series including ST3595 [27]. The ability of ST3595 and related compounds to induce genotoxic stress appears to be cell type-specific, because activation of γH2AX was evident in H460 lung carcinoma cells, but undetectable in ovarian carcinoma cells [17; unpublished observation]. It is conceivable that a mechanism involving alterations of DNA replication is operative also in cells treated with adamantyl retinoids (e.g., ST1926) which exhibits a similar pattern of response [18], thus suggesting a common mechanism of DNA damage by HDAC inhibitors and related atypical retinoids.

3. Sensitization to antimicrotubule agents

The efficacy of HDAC inhibitors in combination with paclitaxel has been reported in various cell systems and in *in vivo* preclinical models [28,29], although the synergistic effect is not a general

event [30,31]. Since epigenetic alterations are associated with the drug-resistant phenotype in tumor types for which taxanes represent effective clinical options, the combination of HDAC inhibitors with paclitaxel has been investigated with the aim to overcome resistance possibly by restoring the apoptotic response [32]. Although the synergistic effect of the combination may reflect enhanced apoptosis, the molecular events involved in the drug interaction remain poorly understood. It is conceivable that the cellular response elicited by the cytotoxic treatment involves up-regulation of protective pathways and HDAC inhibitors could prevent the drug-induced up-regulation of anti-apoptotic factors [29]. Since a variable cellular response to combined treatment of HDAC inhibitors with paclitaxel was observed in different cell lines, the optimization of such combinations requires a better understanding of the critical events implicated in the synergistic interaction. It is likely that the nature of interaction with HDAC inhibitors depends on (a) the biological context (in particular, specific features that may be determinant of cellular response) and (b) on the HDAC isoenzyme specificity of the inhibitor. In addition to the transcriptional effects mediated by histone acetylation, biological effects of HDAC inhibitors may also reflect functional alterations of non-histone proteins implicated in critical regulatory pathways. Hyperacetylation of tubulin has been associated with enhanced induction of apoptosis in endometrial tumor cells treated with trichostatin A and paclitaxel [28]. The efficacy of the combination has been ascribed to the cooperative effects on microtubule stabilization as a consequence of tubulin acetylation. This effect is mediated by inhibition of HDAC6, which in addition to α tubulin deacetylates also Hsp90 and is implicated in misfolded protein stress response [33,17,34]. The cell line-dependent effects of the HDAC inhibitor/paclitaxel combinations do not support a primary role for tubulin acetylation in the synergistic interaction. Recently, we have found that the combination of two structurally related HDAC inhibitors (ST2782 and ST3595) with paclitaxel has a synergistic effect in ovarian carcinoma cells carrying wild-type p53, but not in the p53 mutant platinum-resistant subline, in spite of a marked drug-induced tubulin acetylation (unpublished observation). The efficacy of the combination does not reflect stabilization of microtubules because the synergistic effect was observed also in combination with vinorelbine, a microtubule destabilizing agent. ST2782 prevented taxane-induced p53-dependent up-regulation of p21 which is at least in part p53 dependent and plays a protective role in response to taxane [35]. In keeping with p21 protective role, activation/stabilization of p53 by MDM2 antagonists provides protection from paclitaxel cytotoxicity [36]. In contrast, inhibition of p53 sensitizes p53-proficient tumor cells to antimicrotubule agent-induced apoptosis [35]. The role of modulation of p53 function as a determinant of the synergism between HDAC inhibitors and paclitaxel was supported by lack of sensitization following p53 silencing. Based on the evidence of multiple functions of p53 in response to cytotoxic injury (i.e., protective or pro-apoptotic), the most plausible explanation for the potentiation of the activity of paclitaxel by some HDAC inhibitors is the down-regulation of the expression of protective factors (e.g., p21) and modulation of p53 pro-apoptotic activity. Inhibition of paclitaxel-induced up-regulation of the anti-apoptotic protein, survivin, has been implicated as a mechanism contributing to the sensitization of ovarian carcinoma cells in combination with SAHA [29].

4. Modulation of death receptor pathway and sensitization to TRAIL or agonists

HDAC inhibitors have been regarded as agents capable of reactivating apoptosis in tumor cells, in which apoptosis is frequently impaired [37]. Apoptosis consists of two main distinct pathways, the extrinsic pathway which involves the activation of cell surface death receptors by tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) and death receptor agonists [10,38], and the intrinsic one, which senses and integrates a number of signals of intracellular origin. HDAC inhibitors have been shown to modulate the expression of multiple regulators of apoptotic cell death and, as a consequence, they can trigger apoptosis of tumor cells by interference with both pathways. Overall, several studies have shown the relevance of the death receptor pathway in HDAC inhibitors-induced tumor cell killing both *in vitro* and *in vivo* and the phenomenon appears to occur through up-regulation of ligands and/or receptors of the tumor necrosis factor superfamily [39–41]. The occurrence of tumor-selective apoptosis following exposure to HDAC inhibitors such as MS-275, SAHA or valproic acid (VPA), has been documented in different types of leukemia [40,41]. Induction of the gene coding for TRAIL (TNFSF10) was observed in cells treated with HDAC inhibitors (MS275, SAHA) together with induction of the genes coding for TRAIL Receptor 1 (TRAIL-R1, DR4) and TRAIL Receptor 2 (TRAIL-R2, DR5) [40]. The mechanisms implicated in HDAC inhibitor-mediated activation of TNFSF10 may include (a) inhibition of resident HDACs resulting in activation of promoter-associated factors, (b) recruitment of acetylated transcription factors, (c) displacement of the HDAC complex from the promoter site, (d) induction of events that involve the DNA binding factors Sp1 and Sp3 [40].

Interestingly, VPA and trichostatin A were found to induce up-regulation of TRAIL, TRAIL-R2, FasL and Fas in leukemic cells, but not in preleukemic cells [41]. The extent of modulation of these components of the extrinsic pathway *in vivo* was different for the two HDAC inhibitors, possibly reflecting differential potency or bioavailability of the compounds that may impact on the extent of activation of the various components of the TNF superfamily. Thus, the antitumor effect of HDAC inhibitors can result, at least in part, from sensitization to endogenous TRAIL whose expression can be induced by HDAC inhibitors.

HDAC inhibitors have been reported to enhance the apoptosis-inducing potential of TRAIL in a variety of tumor types [42–45]. A number of studies have shown the capability of such compounds to reactivate the expression of key components of the extrinsic apoptotic pathway. For example, restoration of caspase 8 expression has been achieved in cell systems of medulloblastoma, in which poor prognosis has been associated with loss of caspase 8, following exposure to the combination of HDAC inhibitors with IFN-γ [46]. Such an effect that restored TRAIL-induced apoptosis was due to the capability of HDAC inhibitors to promote histone acetylation and of IFN-γ to stimulate caspase 8 promoter [46]. An analysis of the determinants of sensitization to TRAIL by the HDAC inhibitors romidepsin and LBH589 (panobinostat) in chronic lymphocytic leukemia cells suggests that the critical step in the sensitization process is recruitment of FADD (Fas associated death domain) in the death inducing signalling complex, because treatment with romidepsin did not enhance TRAIL binding to TRAIL-R1 [47]. In hepatocellular carcinoma cells, sensitization to TRAIL by SAHA was related to TRAIL-R2 up-modulation and down-regulation of the caspase 8 inhibitory protein c-FLIP [44].

Overall, the available evidence supports that sensitization to TRAIL can be achieved using HDAC inhibitors, but not a single common mechanism accounts for the combination effects, because different inhibitors regulate various components of the TRAIL signalling pathways [48].

Sensitization to the effects of endogenous/exogenous TRAIL or to TRAIL agonistic antibodies appears an attractive therapeutic strategy by virtue of the rather restricted expression of TRAIL-R1 and TRAIL R2 in tumors, but not in normal cells [49]. Since several tumors have been shown to be resistant to TRAIL receptor agonists

[9,50], the modulation of the death receptor pathway raises the possibility that selective HDAC inhibitors may be useful to overcome resistance to death receptor agonists [45].

Finally, the therapeutic efficacy of HDAC inhibitors does not necessarily require death receptor signalling. In fact, LAQ824 and LBH589 were shown to induce cell death in a transgenic mouse model of pre-B/B-cell lymphoma through engagement of the intrinsic pathway [50]. In this model, tumor cell death was not prevented when the death receptor pathway was blocked, but it was suppressed by overexpression of Bcl-2 or Bcl-x_L. Thus, HDAC inhibitors appear at the crossroad among multiple cell death pathways.

5. Combinations with targeted pharmacological or biological agents

Several preclinical studies have been designed in an attempt to address the interest of combinations of molecularly targeted agents with HDAC inhibitors [12]. In some instances the synergistic interaction has been documented with small molecules or antibodies and it has been related to modulation of specific targets (e.g., reactivation of drug target expression), thus resulting in an enhanced inhibitory effect. An alternative mechanism of synergistic interaction may be independent of target inhibition and may involve modulation of pathways that allow potentiation of the cytotoxic antiproliferative effect, as observed for down-regulation of p21 by protein kinase inhibitors [51,52].

Estrogen receptor (ER)-negative breast tumors have been shown to be sensitized to estrogen and to the aromatase inhibitor letrozole following functional activation of the estrogen receptor alfa and aromatase by MS-275 [53]. Since the lack of expression of ERα in breast cancer has been associated with increased deacetylation [54], a shift from hormone independence to hormone-dependence after treatment with HDAC inhibitors was expected. The hydroxamic acid-based inhibitor LAQ824 was shown to increase apoptosis induced by a variety of agents including trastuzumab in breast cancer cell lines with amplification of Her2 [55]. A striking effect on Her2 levels was observed in such models as a result of decrease of Her2 mRNA levels following HDAC inhibitor treatment and increased degradation by proteasome [56]. The capability of HDAC inhibitors to accelerate decay of mature Erb2 transcripts and to repress new transcript synthesis was identified as a common feature of HDAC inhibitors and proteasome inhibitors in a highthroughput screening carried out using a cell line with stable genomic integration of the Erb2 proximal promoter driving a luciferase reporter [57]. Increased degradation of Erb2 by proteasome was the consequence of acetylation of Hsp90, which impairs its association to client proteins including Her2 [56]. In keeping with these findings, trastuzumab displayed synergism in combination with SAHA in Her2 overexpressing models in which, in addition to the above mentioned effects on Hsp90, HDAC inhibitors produced up-regulation of pro-apoptotic proteins (Bim, Bak) and down-modulation of anti-apoptotic proteins (Bcl-2, Bcl-x_L, XIAP) [55]. Concomitant targeting of HDAC and Her2 appeared to result in increased efficacy also in a cellular context other than breast cancer, i.e., colorectal cancer, as observed with the dual EGF-R/Her2 inhibitor lapatinib and panobinostat [58].

The available evidence supports a broad effect of HDAC inhibitors on survival and proliferation pathways. In fact, in addition to modulating Her2 levels, down-regulation of the ErbB receptor family (EGFR, ErbB2, ErbB3) in terms of expression and signalling by SAHA has been documented [59]. In particular, reversion of epithelial–mesenchymal transition associated with decreased vimentin and increased E-cadherin levels was reported in head and neck squamous cell carcinoma cells which were sensitized to the effect of gefitinib [59]. Moreover, in human acute myelogeneous leukemia cells, LAQ824 in combination with the FLT-3 kinase inhibitor PKC412 promoted proteasomal degradation and attenuation of levels of mutant FLT-3, a receptor tyrosine kinase frequently mutated in such a disease [60].

An alteration of the downstream signalling of mutant KIT has been implicated in the efficacy of the combination of Hsp90 inhibitors and HDAC inhibitors [61]. Consistent with this finding is the report that the pan-HDAC inhibitor AR-42 down-regulates constitutively activated KIT via inhibition of its transcription, thereby leading to increased cell death in malignant mast cell lines [62]. Suppression of KIT signalling by HDAC inhibitors in gastrointestinal stromal tumors was reported to be mediated by Hsp90 acetylation [63].

A major player identified in drug combination studies is represented by the Hsp90 chaperone, which is, as mentioned above, the main substrate of HDAC6 [9]. The inhibition of the chaperone function of Hsp90 following hyperacetylation leads to depletion of pro-survival and anti-apoptotic client proteins [12,64,65]. In a recent report, the natural polyphenol curcumin was shown to enhance the growth inhibitory and apoptotic activity of the pan-HDAC inhibitors SAHA and panobinostat [66]. Such effects achieved at subtoxic concentrations appeared to be mediated by cooperative Hsp90 inhibition by curcumin/HDAC inhibitors, because persistent depletion of Hsp90 client proteins was observed [66]. The improvement of antitumor activity of the mTOR inhibitor temsirolimus by combination with SAHA in renal cell carcinoma models has been ascribed to down-regulation of survivin, an Hsp90 client protein [67]. This combination also resulted in a strong reduction of angiogenesis, a finding which is consistent with a cooperative inhibition of HIF-1α pathway, implicated in the regulation of angiogenesis [68].

Given the role of HDAC6 in processing misfolded proteins via the aggresome pathway [69], the combination of selective HDAC inhibitors and proteasome inhibitors may provide novel therapeutic options [70]. Several studies have already documented the occurrence of synergism between HDAC inhibitors and the inhibitor of the 20S proteasome bortezomib in different tumor models [69]. HDAC6 acts as a microtubule-associated deacetylase endowed with the capacity to bind polyubiquitinated proteins and dinein motors, thus recruiting misfolded proteins to dinein for transport to aggresomes through which proteins are transported from cytoplasm to lysosomes for degradation [71]. A selective sensitization to the effects of combined inhibition of proteasome and HDAC6 has been shown in ovarian cancer cells as a result of activation of ubiquitin proteasome system stress [72].

The efficacy of these combinations appears to be related to multiple mechanisms and in part dependent on the cell background. In uterine cervical cancer, where the E6 and E7 oncogenes maintain the transformed phenotype, a synergistic interaction was observed when combining bortezomib with the pan-HDAC inhibitors SAHA and thricostatin A, but not with the HDAC6 specific inhibitor tubacin [73]. Since the oncoprotein E7 interacts with HDAC1 and HDAC2, the synergistic killing appeared to be mediated by such isoforms. Modulation of the proteasome function may result in increased apoptotic cell death. Such an event may be favoured in the presence of mutant p53 [74]. In fact, we recently found that the synergistic interaction observed in ovarian carcinoma cells treated with the HDAC inhibitor ST2782 and bortezomib was more evident in p53 mutant platinum resistant sublines as compared with sensitive cells carrying wild-type p53 [unpublished observations]. Interestingly, p53 acetylation has been shown to be crucial for transcription-independent proapototic effects [74]. Conversely, SAHA has been recently shown to be endowed with post-translational effects, not mediated by epigenetic mechanisms, because it could stimulate

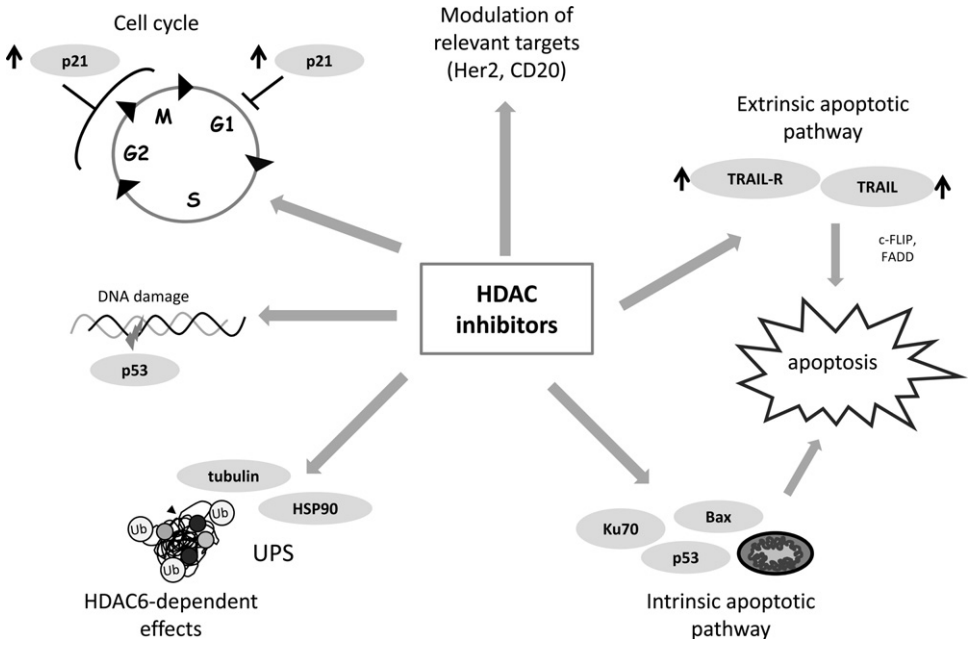

Fig. 1. Possible mechanisms by which HDAC inhibitors exert biological activities which may be relevant for the interaction with other antitumor agents. The mechanisms include modulation of pathways which are dependent on or independent of histone acetylation. Hyperacetylation of histones may result in (a) modulation of expression of genes responsible for cell cycle arrest or regulation of pathways implicated in activation of apoptosis; (b) changes in the expression of proteins relevant as therapeutic targets. Acetylation of non-histone proteins (including transcription factors) cooperates in the proapoptotic effects and may be implicated in cellular response to genotoxic stress. HDAC inhibitors may also induce the formation of reactive oxygen species (ROS) resulting in oxidative DNA damage.

the degradation of mutant p53 [75]. Interestingly, the down-regulation of selected proteasome subunits after exposure of ovarian carcinoma cells to a HDAC inhibitors structurally related to atypical retinoids has been observed using a proteomic approach [76]. Although the effect on proteasome activation is not documented, it is likely that such a treatment results in proteasome activity impairment.

Finally, the current interest of HDAC inhibitors in the treatment of lymphoma has stimulated the development of rational combinations of HDAC inhibitors with other clinically effective agents [77,78]. In this context, relevant is the finding that in lymphoma cells up-regulation of CD20 expression by the HDAC inhibitors, VPA or romidepsin, resulted in increased cytotoxic activity of the anti-CD20 antibody rituximab [79]. The effect was observed in human lymphoma cells displaying modest expression of the CD20 antigen. The molecular mechanism of increased CD20 expression has been linked to Sp1 recruitment following CD20 gene promoter hyperacetylation. The modulation of CD20 expression may have obvious implications for optimization of rituximab therapy.

6. Discussion

Epigenetic changes are associated with tumor progression and may involve the down-regulation of growth regulatory and apoptotic pathways [6]. Epigenetic modifications have been also implicated in a reversible drug-tolerant state following drug treatment [80]. The growing evidence that epigenetic changes plays a relevant role in tumor progression and may be involved in acquiring drug resistance of tumor cells has stimulated efforts to develop intervention strategies targeting HDAC. The therapeutic efficacy of HDAC inhibitors has been ascribed to their ability to induce apoptosis, which could reflect multiple mechanisms, not only the reversing of epigenetic events.

Although HDAC inhibitors exhibit antitumor activity as single agents in preclinical studies, it is now evident that their therapeutic potential could be better exploited in rationally designed combinations with conventional or novel targeted agents [12,81]. A relatively simplistic explanation for the enhanced efficacy of HDAC inhibitors in combination with other agents is their ability to lower the apoptotic threshold. However, there is a strong molecular rationale for various combination strategies using targeted agents, because HDAC inhibitors may modulate the expression or function of cancer-relevant specific targets or pathways (Fig. 1). It is likely that, given the pleiotropic effects of pan-HDAC inhibitors, multiple mechanisms converge to confer sensitization of tumor cells to conventional cytotoxic agents, in particular to DNA damaging agents. HDAC inhibitors could potentiate the effects of genotoxic agents by enhancing DNA damage response or by interfering with DNA repair processes [12]. The ability of some HDAC inhibitors to induce genotoxic stress could contribute to the chemosensitizing effect [16,82]. The mechanism(s) for induction of DNA damage is not clearly understood, also taking into account that HDAC6-selective inhibitors exhibit DNA-damaging effects in spite of non-histone deacetylation activity recognized for HDAC6 [16].

The interest for HDAC6 inhibitors is not solely related to their chemosensitizing potential, but also to the role of HDAC6 in cancer-relevant processes [9,83,84] (Fig. 2). By deacetylating Hsp90, HDAC6 has been implicated in the modulation of survival and anti-apoptotic factors, including EGFR, Akt and survivin [85]. In addition, HDAC6 is recognized as a critical regulator of epithelial–mesenchymal transition [86], a process contributing to drug resistance [87,88]. HDAC6 also plays a role in regulation of intracellular interactions and cell migration [89]. Indeed, agents effective as HDAC6 inhibitors exhibit antimetastatic activity [90,91]. HDAC6 has been implicated in aggresome pathway and degradation of misfolded proteins, by regulating the acetylation state of Hsp90. This function could be rationally exploited in combination with Hsp90 inhibitors and with agents which induce stress response [70]. HDAC6 is involved in the stabilization of aberrant androgen receptor (AR) levels, directly or indirectly via Hsp90 and in the ligand-dependent activation of this receptor. Since the overexpression/mutation and/or posttranslational modification of AR or its coactivators cause the progression to an

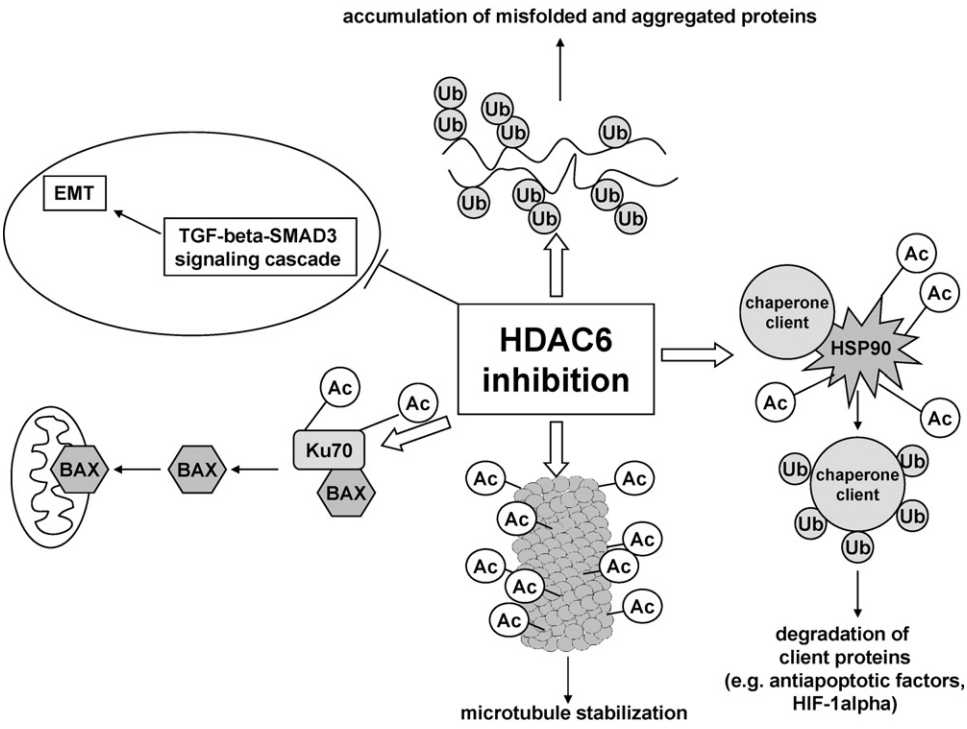

Fig. 2. Schematic overview of HDAC6 inhibition effects. HDAC6 inhibition causes an accumulation of toxic misfolded/aggregated proteins. HDAC6 controls the acetylation status of Hsp90 and of α-tubulin. Hsp90 hyperacetylation disrupts its chaperone function leading to depletion of pro-survival, anti-apoptotic and angiogenic client proteins. α-Tubulin acetylation favours microtubule stabilization. HDAC6 forms a complex with cytoplasmic Ku70 and inhibition of HDAC6 activity increases Ku70 acetylation, resulting in Bax release and induction of mitochondria-dependent apoptotic cell death. HDAC6 inhibition may also result in reversion of epithelial to mesenchymal transition.

androgen-independent state, the use of HDAC6 inhibitors in prostate cancer could be an effective strategy to counteract alterations responsible for the AR-resistant phenotype [92]. Thus, based on multiple functions, HDAC6 appears to be a potential target to be exploited to improve the efficacy of various antitumor agents. Given the good tolerability, selected HDAC6 inhibitors [27,90] appear more suitable for their use in combination therapy, because of the predicted lack of major side effects. Indeed, the recently reported HDAC inhibitors with preferential inhibitory activity against HDAC6 exhibit a good profile of tolerability [27].

A large number of options can be envisaged to improve antitumor therapies with HDAC inhibitors [93]. It is conceivable that the choice of HDAC inhibitor combined with other agents to achieve synergistic interaction is dependent on the mechanism of action and cellular response to drug treatment. In addition to DNA damaging agents, promising results have been reported in combination of various HDAC inhibitors with taxanes, DNA methyl transferase inhibitors, hormonal therapy and target-specific agents [93]. Additional combinations endowed with therapeutic interest may include agents that stimulate the production of TRAIL and other cytokines within the tumor or immunological stimulants [46,94,95]. HDAC inhibitors have been shown to sensitize human pancreatic cancer cells to IL-13 receptor targeted immunotoxin and to enhance the antitumor activity of a DNA vaccine driven by a CMV promoter [95]. HDAC inhibitors have also been reported to inhibit tumor-induced angiogenesis, an effect mediated by HIF-1α down-regulation and modulation of pro-angiogenic factors [96,97]. This observation suggests therapeutic opportunities for combination of HDAC inhibitors with various anti-angiogenic agents.

Relevant to the combination strategies is the observation that normal cells are less sensitive than tumor cells to the antiproliferative and pro-apoptotic activities of HDAC inhibitors. This accounts for the relatively good therapeutic index of inhibitors. Specifically, targeting multiple pathways with safe combinations designed to induce synergistic effects is an attractive therapeutic option. Based on the preclinical evidence of potentially useful interactions, several clinical studies have been designed [12,34,81]. The mechanisms of these interactions have not been completely elucidated. Thus, in this approach, the optimal strategy with the use of pan-HDAC or isoenzyme-specific inhibitors remains to be defined. A better knowledge of the role of individual HDAC isoforms in oncogenic pathways and malignant behaviour, and the development of selective HDAC inhibitors could overcome the limitations of empirical selection of combinations. The identification of novel HDAC inhibitors with more isoenzyme-specific inhibition is expected to provide useful tools to better exploit synergistic interactions.

In conclusion, the complexity of genetic alterations and the development of multiple resistance mechanisms suggest that an effective control of tumor growth requires combination therapies to optimize the outcome of treatment. The achievement that, in addition to genetic alterations, epigenetic modifications contribute to drug resistance, in particular to the onset of acquired resistance, provides the basis for the design of novel treatment strategies aimed at enhancing tumor cell death susceptibility and at preventing adaptive changes during therapy. The development of HDAC inhibitors provides an additional opportunity to explore the efficacy of inhibition of multiple cancer-related pathways and the relevance of interference with resistance mechanisms in specific biological contexts. The optimization of the use of HDAC inhibitors requires a better understanding of the role of each HDAC isoforms as therapeutic targets for various combinations. The development of isoenzyme-selective inhibitors is expected to improve the rational basis for the incorporation of HDAC inhibitors in cancer therapy.

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