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The histone deacetylase inhibitor suberic bishydroxamate: a potential sensitizer of melanoma to TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis

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Abstract

TRAIL appears to be a promising anticancer agent in that it induces apoptosis in a wide range of cancer cells but not normal tissues. Sensitivity of melanoma cells to TRAIL-induced apoptosis varied considerably because of their development of various resistance mechanisms against apoptosis. We discuss in this report the potential effect of a histone deacetylase inhibitor SBHA on TRAIL-induced apoptosis. Histone deacetylase (HDAC) inhibitors regulate histone acetylation and thereby modulate the transcriptional activity of certain genes leading to cell growth arrest, cellular differentiation, and apoptosis. Suberic bishydroxamate (SBHA) is a relatively new HDAC inhibitor that induced apoptosis in the majority of melanoma cell lines through a mitochondrial and caspase-dependent pathway. This was due to its regulation of the expression of multiple proteins that are involved in either the mitochondrial apoptotic pathway (Bcl-2 family members) or the final phase of apoptosis (caspase-3 and XIAP). Co-treatment with SBHA at nontoxic doses and TRAIL resulted in a marked increase in TRAIL-induced apoptosis of melanoma, but showed no toxicity to melanocytes. SBHA appeared to sensitize melanoma to TRAIL-induced apoptosis by up-regulation of pro-apoptotic proteins in the TRAIL-induced apoptotic pathway such as caspase-8, caspase-3, Bid, Bak, and Bax, and up-regulation of the BH3 domain only protein, Bim. This, together with activated Bid, may have acted synergistically to cause changes in mitochondria. Treatment with SBHA also resulted in down-regulation of antiapoptotic members of the Bcl-2 family, Bcl-X_L and Mcl-1, and the IAP member, XIAP. These changes would further facilitate apoptotic signaling. SBHA appeared therefore to be a potent agent in overcoming resistance of melanoma to TRAIL-induced apoptosis.

Keywords: Melanoma; Histone deacetylase inhibitors; Suberic bishydroxamate; Apoptosis; TRAIL

1. Introduction

Histones are basic proteins that, by complexing with DNA, form nucleosomes leading to the compact structure of chromatin. Basic amino acids of the histones can be post-translationally modified with methyl, acetyl or phosphate groups. The balance between histone acetylation and deacetylation is regulated by histone acetyltransferase (HAT) and HDACs, and plays an important role in transcriptional regulation of genes [1–5]. Acetylation of lysine residues of histones results in more open chromatin structure and therefore activation of transcription, whereas hypoacetylation of histones is associated with a condensed chromatin structure resulting in the repression of gene transcription [4,5]. Deregulation of HAT and HDACs has been implicated in the formation and development of certain human cancers by changing the expression pattern of various genes [6–9].

HDACs are a family of enzymes that regulate histone acetylation by catalyzing the removal of acetyl groups on lysine residues of the nucleosomal histones [4,5]. HDAC inhibitors are members of a class of agents that modulate the expression of genes by inhibiting the HDAC activity,

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Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; SBHA, suberic bishydroxamate; HDAC, histone deacetylases; IAP, inhibitor of apoptosis proteins; c-IAP, cellular inhibitor of apoptosis; XIAP, X-linked IAP; ICAD, inhibitor of caspase-activated DNase; PARP, poly(ADP-ribose) polymerase; FLIP, FLICE/caspase-8 inhibitory protein; MAb, monoclonal antibody; Ab, antibody; FLICE, FADD-like interleukin-1 converting enzyme.

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resulting in an increase in histone acetylation [4,5]. DNA microarray studies using malignant cell lines cultured in the presence of a HDAC inhibitor showed that only a small number (1–2%) of genes were regulated [10]. The altered gene expression after exposure to a HDAC inhibitor has been demonstrated to arrest cell growth [11–13] and to reverse neoplastic characteristics by inducing differentiation [14–17]. In addition, HDAC inhibitors can induce apoptotic cell death in a variety of tumor cell lines [14,18–20]. HDAC inhibitors are therefore considered to be promising chemotherapeutic agents for treatment of malignant tumors. The potential significance of HDAC inhibitors as anticancer agents has been supported by studies in animal models and clinical trials showing antitumor activity with minor toxicity to normal tissues *in vivo* [21,22].

Although a number of HDAC inhibitors have been shown to induce apoptosis of cultured tumor cells, the mechanism(s) underlying this appear to vary. For example, explanations for the induction of apoptosis by the non-specific HDAC inhibitor sodium butyrate include alterations in Bcl-2 family protein expression [23–26], increased caspase activity [20], increased sensitivity to Fas–Fas ligand interaction [27,28] and changes in the expression of genes such as c-*myc* and k-*ras* [29]. Moreover, caspase-independent mechanisms have also been suggested as causative [25]. It appeared that sodium butyrate-induced apoptosis was mediated through the mitochondrial pathway in that apoptosis was inhabitable by overexpression of Bcl-X_L [25]. Studies with another HDAC inhibitor, apicidin, showed that this was associated with translocation of Bax to mitochondria and subsequent release of cytochrome *c* [30]. The HDAC inhibitor suberoylanilide (SAHA) appeared to induce apoptosis by direct noncaspase-dependent activation of Bid [31].

Much interest has been given to the role of HDAC inhibitors in regulation of $p21^{WAF1/CIP1}$ [19,32,33], which is one of the key regulators of the cell cycle. The transcription of $p21^{WAF1/CIP1}$ is up-regulated in response to HDAC inhibitors as a consequence of increased acetylation of the chromatin at the Sp1 binding sites in the promoter region of $p21^{WAF1/CIP1}$ [33,34]. Up-regulation of $p21^{WAF1/CIP1}$ by HDAC inhibitors plays a critical role in cell cycle arrest by inhibiting cyclin-dependent kinase (cdk) activity [13,32]. It may also participate in the induction of apoptosis in that overexpression of $p21^{WAF1/CIP1}$ leads to induction of cytochrome *c* release, activation of caspases, and apoptosis [33,34]. However, cell lines that did not have increased $p21^{WAF1/CIP1}$ protein levels in response to the HDAC inhibitor acelaic bishydroxamic (ABHA) were more sensitive to apoptosis than cell lines in which $p21^{WAF1/CIP1}$ protein levels were increased following treatment with ABHA [19].

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family that can induce apoptosis in a wide range of cultured malignant cells including melanoma [35–38]. The potential significance of TRAIL as an anticancer agent has been supported by studies in animal models showing selective toxicity to human tumor xenografts but not normal tissues. Traditionally, two principal pathways to apoptosis have been recognized, the transmembrane ''extrinsic'' pathway and the mitochondrial ''intrinsic'' pathway. Both depend on activation of cysteine proteases that cleave at aspartate residues (caspases). These enzymes are synthesized as proenzymes that become activated by adaptor proteins. Initiator caspases once activated can activate so-called effector caspases, which act on a wide range of substrates to cause apoptosis. The initiator caspases for the extrinsic pathway are caspase-8 and -10, whereas caspase-9 and -2 are initiator caspases for the intrinsic pathway. The effector caspases are believed to be similar for both pathways, i.e. 3, 6 and 7 [39,40]. TRAIL-induced apoptotic signaling is believed to be initiated by ligand-induced aggregation of death domains that reside on the cytoplasmic sides of the death receptors, TRAIL-R1 and -R2. This in turn orchestrates the assembly of adaptor proteins such as Fas-associated death domain (FADD) that activate initiator caspases, caspase-8 and -10 leading eventually to activation of effector caspases such as caspase-3 [37,41,42].

TRAIL-induced apoptosis of melanoma is largely dependent on induction of changes in mitochondrial membrane permeability [43,44]. Release of Smac/DIABLO from mitochondria to the cytosol mediates TRAIL-induced apoptosis by binding to members of the inhibitors of apoptosis protein (IAP) family. This prevents the IAP proteins binding to the effector caspase-3 and -4, and to caspase-9, so allowing apoptosis to proceed [39,40,43]. TRAIL induces low levels of Smac/DIABLO release into the cytosol in TRAIL resistant melanoma cell lines but high levels in TRAIL sensitive cell lines [43]. The basis for this variation in Smac/DIABLO release remains largely unknown but did not appear to be due to differences in activation of caspase-8 and Bid in that they appeared to be cleaved to the same extent in both TRAIL sensitive and resistant melanoma cell lines [43]. It was however suggested that Bcl-2 family members play a pivotal role in regulating mitochondrion-mediated apoptosis. The antiapoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-X_L and Mcl-1, appear to preserve the integrity of the mitochondrial outer membrane by binding to BH3 domains of the pro-apoptotic BH3-only proteins such as Bid, Bim, and Noxa [45,46]. In the absence or deficiency of the antiapoptotic Bcl-2 family members, the pro-apoptotic BH3-only proteins cause the oligomerization of the multi-BH3 domain proteins, Bax and Bak, in the mitochondrial outer membrane and formation of pores which allow release of mitochondrial proteins such as Smac/DIABLO and cytochrome *c* [47]. A schematic illustration of TRAIL-induced apoptosis pathway in melanoma is shown in Fig. 1.

SBHA is a relatively new HDAC inhibitor that can induce apoptosis in the majority of melanoma cell lines through a mitochondrial and caspase-dependent pathway [48].

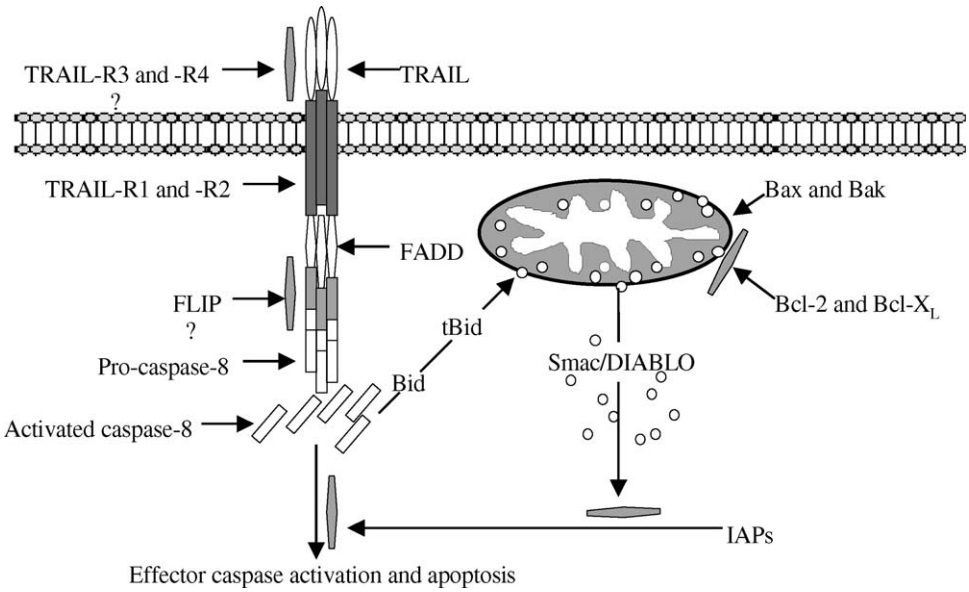

Fig. 1. A schematic illustration of TRAIL-induced apoptotic pathway in melanoma cells. TRAIL induces aggregation of death domains that reside on the cytoplasmic sides of the death receptors that in turn recruit adaptor proteins such as FADD. The latter bind the prodomains of caspase-8, activation of which results in activation of caspase-3 and cleavage of Bid. The truncated Bid targets mitochondria and causes relocation of Bax and Bak from the cytosol to mitochondria leading to changes in mitochondrial membrane permeability and release of Smac/DIABLO to the cytosol, where Smac/DIABLO binds to inhibitors of apoptosis such as XIAP and frees activated caspase-3 from the latter leading to apoptotic execution. TRAIL-R3 and -R4 are believed to act as decoy receptors and FLIP is thought to compete with caspase-8 for binding to FADD and inhibits its activation. The antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_L can inhibit changes in mitochondria and release of mitochondrial apoptotic proteins such as Smac/DIABLO.

Several key antiapoptotic proteins are down-regulated in SBHA-treated cells [49]. These include X-linked IAP (XIAP) and the Bcl-2 family proteins, Mcl-1 and Bcl-X_L. In contrast, SBHA induces up-regulation of the Bcl-2 family pro-apoptotic proteins, Bid, Bim, Bax, and Bak. Moreover, the expression levels of pro-caspase-8 and pro-caspase-3 are also up-regulated by SBHA treatment. This combination of events strongly suggests that SBHA may be a valuable agent in sensitizing melanoma to other apoptosis inducers. In this report, we discuss the possible mechanisms by which SBHA induces apoptosis and the potential effect of SBHA on TRAIL-induced apoptosis of melanoma.

2. SBHA-induced apoptosis of melanoma is caspase-dependent

Studies in a panel of melanoma cell lines have shown that SBHA induces apoptosis in the majority of the cell lines with relatively slow kinetics [49]. The percentage of apoptotic cells peaked between 24 and 48 hr after SBHA treatment. There was a wide variation in sensitivity of melanoma cells to SBHA-induced apoptosis, with negligible cell death in a few cell lines but nearly 50% apoptotic cells in some others. SBHA did not induce apoptosis in melanocytes even when the cells were treated for prolonged periods. Apoptosis of melanoma induced by SBHA appeared to be caspase-dependent in that the pan-caspase inhibitor, z-VAD-fmk, completely inhibited cell death [49]. A caspase-3 specific inhibitor, z-DEVD-fmk or a caspase-9 specific inhibitor, z-LEHD-fmk also blocked SBHA-induced apoptosis. The involvement of the caspase cascade in SBHA-induced apoptosis was confirmed by the findings that both caspase-3 and caspase-9 were activated in cells after exposure to SBHA. The pro-enzyme form of caspase-3 appeared to undergo up-regulation shortly after SBHA treatment in that an increase in the expression levels was detected that was followed by slow reduction. The critical role of the caspase cascade in SBHA-induced apoptosis was further supported by the observations of cleavage of caspase-3 substrates inhibitor of caspase-activated DNase (ICAD) and poly(ADP-ribose) polymerase (PARP). In contrast to capase-3 and caspase-9, pro-caspase-8 was up-regulated without any degradation and appearance of the active form of the enzyme, suggesting the death receptor pathway may not be involved in SBHA-induced apoptosis of melanoma [49].

3. SBHA-induced apoptosis of melanoma is dependent on changes in mitochondria

The involvement of capsase-9 but not caspase-8 in SBHA-induced apoptosis suggested that SBHA may be acting to induce changes in mitochondrial membrane permeability (MMP) leading to the release of mitochondrial apoptotic proteins [49]. This was demonstrated by reduction of mitochondrial membrane potential ($\Delta\Psi_m$) and release of cytochrome *c* and Smac/DIABLO from mitochondria into the cytosol after exposure to SBHA.

Further evidence in support of the role of mitochondria in SBHA-induced apoptosis came from studies on Bcl-2 overexpressed cells. The levels of SBHA-induced apoptosis in Bcl-2 transfectants were markedly decreased, so were SBHA-induced changes in the $\Delta\Psi_m$, caspase-3 activation, and cleavage of ICAD and PARP [49].

Bax translocation from the cytosol to mitochondria is believed to play a key role in induction of apoptosis by a variety of apoptotic stimuli [50]. Bax translocation involves a conformational change that exposes the NH_2-terminus and the hydrophyobic COOH-terminus that targets mitochondria [51,52]. The NH_2-terminal region is occluded in intact cells and hence is not available for binding by Bax-, NH_2-terminal epitope-specific antibodies [53,54]. Studies on conformation status of Bax in melanoma cells with or without exposure to SBHA by using an antibody directed against the NH_2-terminal region of Bax showed an increase in the Bax conformation change after SBHA treatment with kinetics that was consistent with the kinetics of apoptosis. The degree of increase in the Bax conformation change appeared to be correlated with the levels of apoptosis induced by SBHA. The mechanisms underlying the change in Bax conformation appeared to be independent of the caspase cascade in that the change in Bax conformation was up-regulated after exposure to SBHA irrespective of inhibition of caspase activity [49]. This indicates that the change in conformation of Bax may be upstream of changes in MMP and activation of caspases.

4. SBHA down-regulates the expression of the antiapoptotic Bcl-2 family members and XIAP, but up-regulates the expression of pro-apoptotic Bcl-2 family members

Examination of Bcl-2 family proteins and XIAP expression in melanoma cells with or without exposure to SBHA showed that SBHA down-regulates the levels of expression of XIAP and the antiapoptotic Bcl-2 family members, Bcl-X_L and Mcl-1, but up-regulates the multi-domain pro-apoptotic proteins, Bax and Bak, and BH-3 only proteins, Bid and Bim [49]. Studies by real time PCR analysis showed that alterations in Bcl-2 family protein expression may be due to the regulation of transcription by SBHA in that the level of Bcl-X_L mRNA expression was down-regulated but the level of Bax mRNA expression was up-regulated after treatment with SBHA [49].

The mechanism by which SBHA regulates such a diverse array of genes involved in apoptosis in melanoma is not clear. It is known that histone deacetylases (HDAC) act as repressors or co-activators of known transcription factors. It is therefore conceivable that SBHA may target histone deacetylases associated with transcription factors that are involved in regulation of apoptosis, such as p53 or c-*myc*. If p53 was involved it might be expected that SBHA would not induce apoptosis in cells containing mutated p53 but would do so in cells with wild-type p53. This did not appear to be the case in that some melanoma cells with mutated p53 were sensitive whereas others with wild-type p53 were resistant to SBHA-induced apoptosis. In support of this, induction of Noxa could not be detected in melanoma cells treated with SBHA. Further study of the role of p53 is however needed. The target genes involved in apoptosis associated with c-*myc* are not well defined [55]. The c-*myc* overexpression was associated with the conformational change in Bax but not with an increase in Bax mRNA [56]. Changes in expression of the Bcl-2 or IAP family have so far not been linked to c-*myc* expression so it seems unlikely that SBHA is acting to repress or activate this particular transcription factor. Further studies using DNA microarrays are now needed to further explore the effects of SBHA on gene transcription in melanoma.

5. Possible initiating factor(s) in SBHA-induced apoptosis of melanoma

It is generally accepted that apoptosis acting *via* the mitochondrial pathway is dependent on the balance between pro-apoptotic BH3-only Bcl-2 family members and the antiapoptotic Bcl-2 family members. The pro-apoptotic BH3 proteins include Bid (activated by caspase-8), Bim or Bmf (transcriptionally regulated or released from damage to microtubules) [57] or the actin myoskeleton [58], and Noxa and PUMA that are up-regulated by gene transcription resulting from activation of p53 [59,60]. The antiapoptotic proteins, Bcl-2 and to a lesser extent Bcl-X_L, are bound to mitochondrial and other membranes in the cells, and bind any BH3-only proteins released into the cytosol [61]. Should the concentration of BH3 proteins exceed that of the antiapoptotic proteins, the multi-domain proteins, Bax and Bak undergo changes in conformation [62] that allow them to aggregate in mitochondrial outer membranes and induce release of apoptotic proteins, such as Smac/DIABLO and cytochrome *c*.

Given the large number of proteins in the Bcl-family, it was quite remarkable that treatment with SBHA resulted in up-regulation of several pro-apoptotic proteins. At the same time, there was a marked decrease in the antiapoptotic Bcl-X_L and Mcl-1 proteins. There was also a marked down-regulation of XIAP, which is an important inhibitor of active caspase-3 in melanoma cells [43]. The kinetics of these changes after exposure to SBHA was consistent with the kinetics of apoptosis. It is possible that SBHA induces an excess of BH-3 only proteins (Bim) over that of Bcl-2 antiapoptotic proteins (Bcl-X_L and Mcl-1), and thereby provides the conditions needed for induction of conformational changes in Bax and induction of changes in mitochondrial membrane permeability and apoptosis.

Consistent with this, the conformational change in Bax was not dependent on caspase activation, as would be seen in caspase-8 activation of Bid or activation of caspases

downstream of changes in MMP. The events initiating conformational change in Bax were therefore upstream of changes in mitochondria and not dependent on the latter. It is also consistent with failure to detect activation of Bid by SBHA. Although Bim can be released from microtubules by agents such as Taxol, it is also transcriptionally regulated. Up-regulation of Bim by deprivation of growth factors depends on the forkhead transcription factor, FKHR-L1 [63,64]. In other systems activation of Jun-terminal kinase (JNK) was involved [65].

6. Relation of regulation of p21 by SBHA to SBHA-induced apoptosis of melanoma

Previous studies have shown an association between treatment with HDAC inhibitors, p21 expression, cell cycle arrest and apoptosis. Studies on melanoma cell lines treated with SBHA showed that p21 was up-regulated in all the cell lines with nonmutated p53 up-regulation of p21 by HDAC inhibitors however may be independent of p53 and result from increased acetylation of the promoter regions of 21 [33]. The role of p21 in induction of apoptosis is controversial. Up-regulation of p21 was found to inhibit apoptosis in response to chemotherapy [66,67], and to a HDAC inhibitor [19]. In contrast, overexpression of p21 in ovarian carcinoma cells induced apoptosis [68] and optimal induction of apoptosis in mouse fibroblasts by ketocholesterol required p21 expression [69]. In our studies on melanoma cells exposed to SBHA there was no apparent relationship between p21 expression and induction of apoptosis [49]. This suggests that other factors are more important in determining the apoptotic response to SBHA than induction of p21. The role of p21 in the antiproliferative effects of HDAC inhibitors however appears well established [70,71].

7. SBHA sensitizes melanoma to TRAIL-induced apoptosis

Given that SBHA appears to regulate a large number of apoptosis mediators involved in either the mitochondrial apoptotic pathway (Bcl-2 family members), the death receptor pathway (caspase-8 and Bid), or the final common pathway to apoptosis (caspase-3 and XIAP), it could be predicted to sensitize melanoma cells to other apoptosis inducers, such as TRAIL which acts *via* these pathways. This proved correct in that co-treatment of melanoma cell lines with SBHA and TRAIL at optimal doses for induction of apoptosis resulted in either synergistic or additive effects on induction of apoptosis in melanoma [49]. Although neither SBHA nor TRAIL induced apoptosis of melanocytes, about one-third of melanocytes underwent apoptosis when treated with SBHA together with TRAIL. Further studies showed that at 30 times lower doses, SBHA did not induce apoptosis alone, nor did it induce caspase activation or changes in mitochondrial membrane permeability in any of the melanoma cell lines. It retained however the ability to sensitize cells to TRAIL-induced apoptosis. There was no toxicity to melanocytes when SBHA was used at lower doses together with TRAIL [49].

8. Possible mechanisms underlying sensitization of melanoma cells to TRAIL-induced apoptosis by SBHA

We examined whether SBHA sensitized melanoma cells to TRAIL-induced apoptosis by up-regulation of TRAIL-death receptor expression [37]. Studies before and after treatment with SBHA at various doses for different time periods failed to show any significant difference in the levels of TRAIL-R1 and TRAIL-R2 death receptor expression [49]. We next examined whether SBHA may overcome intracellular resistance mechanisms against TRAIL-induced apoptosis [43]. One such mechanism is inhibition of caspase-8 interaction with the adaptor protein FADD by a protein called cellular FLICE/caspase-8-inhibitory protein (c-FLIP). However, we have previously shown that there is no correlation between the levels of c-FLIP expression and TRAIL-induced apoptosis of melanoma [38,43]. Some cell lines with high levels of c-FLIP expression were sensitive, whereas others with no or little c-FLIP expression were resistant to TRAIL-induced apoptosis [38,43]. It is therefore unlikely that regulation of c-FLIP by SBHA accounts for its sensitization of melanoma cells to TRAIL.

Our studies showed that the pan-caspase inhibitor z-VAD-fmk completely inhibited TRAIL-induced apoptosis in the presence of SBHA. Furthermore, in the presence of SBHA, TRAIL induced an increase in caspase-3 activation and cleavage of its substrates ICAD and PARP. Similarly, an increase in the reduction of the $\Delta\Psi_m$ and release of cytochrome *c* and Smac/DIABLO induced by TRAIL was observed in the presence of SBHA. This suggests that SBHA may act at the level of mitochondria to sensitize melanoma cells to TRAIL. In support of this, overexpression of Bcl-2 blocked TRAIL-induced apoptosis and the increase in apoptosis in the presence of SBHA.

Given that SBHA up-regulates a number of pro-apoptotic proteins and down-regulates several antiapoptotic proteins, it is probable that sensitization to TRAIL-induced apoptosis may occur at various stages in the TRAIL-induced apoptotic pathway in melanoma cells. Firstly, up-regulation of pro-apoptotic proteins in the TRAIL-induced apoptotic pathway such as pro-caspase-8, pro-caspase-3, Bid, Bak, and Bax would be expected to amplify changes in mitochondrial membrane potential (MMP) induced by TRAIL. Secondly, up-regulation of the BH3-only protein Bim may act synergistically with truncated Bid (tBid) to enhance changes in MMP. Thirdly, down-regulation of the antiapoptotic Bcl-2 family members Bcl-X_L and Mcl-1

may increase the available concentrations of tBid and Bim, and enhance changes in MMP leading to greater release of apoptotic proteins from mitochondria. Fourthly, down-regulation of XIAP may decrease its inhibition of active caspase-3 leading to enhanced processing of its substrates [43].

Past studies have shown that caspase-9 activation did not appear to play a major role in TRAIL-induced apoptosis of melanoma in that its activation could not be detected after TRAIL treatment and a specific inhibitor of caspase-9 had negligible effects on TRAIL-induced apoptosis [43,72]. This is believed to be due to low expression of Apaf-1, which forms the apoptosome together with cytochrome *c*, leading to activation of caspase-9 [73]. Moreover, cytochrome *c* release from mitochondria after exposure of melanoma cells to TRAIL appears to occur relatively late and peak well after the time of maximal activation of caspase-3 [43]. In contrast, studies on SBHA-induced apoptosis and its sensitization of melanoma to TRAIL-induced apoptosis showed that caspase-9 activation was involved in that inhibition of caspase-9 activation markedly blocked apoptosis in the former, and to a lesser extent in the latter. This indicates that the apoptosome was formed in the presence of SBHA, and suggests that Apaf expression may be restored in melanoma cells after treatment with SBHA. Inactivation of Apaf-1 is believed to be due to transcriptional repression due to methylation of its promoters [73]. HDACs play an important role in gene silencing arising from methylation because methylated CpG islands in promoter regions are recognized by proteins that recruit histone deacetylases, leading to stable transcriptional repression [74]. It is therefore conceivable that SBHA may inhibit the effect of methylation of the promoter regions of Apaf-1 gene by blocking histone deacetylation, leading to restoration of Apaf-1 transcription. This explanation however needs to be verified by study of Apaf-1 expression levels with or without exposure to SBHA.

9. Conclusion and prospects

The anticancer potential of HDAC inhibitors has been widely acknowledged [11–22]. This is believed to be at least partially due to their ability to induce apoptosis [14,18–20]. Although a wide range of mechanisms has been suggested, it remains controversial as to how HDAC inhibitors induce apoptotic cell death [14,18–20]. The studies presented here suggest that the HDAC inhibitor SBHA induces apoptosis of melanoma through multiple mechanisms, involving regulation of the expression of the apoptosis mediators as summarized in Table 1. However, studies using antisense or RNA interference techniques are needed to examine which protein is the most important in determining sensitivity of melanoma to SBHA-induced apoptosis.

Table 1
SBHA regulates the expression of multiple pro- and antiapoptotic proteins

	Bcl-X_L	↓*
	Mcl-1	↓
	Bid	↑
Bcl-2 family members		
	Bim	↑
	Bax	↑
	Bak	↑
Caspases	Pro-caspase-8	↑
	Pro-caspase-3	↑
IAPs	XIAP	↓

* ↓ indicates the level of expression was down-regulated; ↑ indicates the level of expression was up-regulated.

Resistance to apoptosis has been reported in many forms of tumors including melanoma [43,72]. Similarly, aberrant HDAC activity has been increasingly shown to be associated with cancer development [6–9]. The large number of apoptosis-related proteins regulated by SBHA suggests that genes encoding these proteins are particularly sensitive to inhibition of HDACs [10]. It is therefore possible that defects in histone acetylation/deacetylation may underlie altered transcription of these genes and resistance to apoptosis. It is notable in this regard that pro-apoptotic proteins are generally up-regulated, whereas antiapoptotic proteins are generally down-regulated by SBHA. This suggests that histone hypoacetylation may be one important cause for the resistance of melanoma to apoptosis. HDACs may however deacetylate other proteins such as p53 and heat shock protein, so that the effect of the HDAC inhibitors may not necessarily always result from effects on histone acetylation [75]. It is unclear if regulation of such a large number of apoptotic mediators is particularly characteristic of SBHA, or whether other HDAC inhibitors also possess this ability. Similarly, it remains unknown if SBHA can regulate the expression of apoptosis mediators in other tumor types. Further studies are needed to address these questions.

TRAIL appears to be a promising agent in treatment of melanoma in that it induces apoptosis in about two-thirds of melanoma cell lines [38,43,72]. However, there is a wide variation in sensitivity of melanoma to TRAIL-induced apoptosis due to various resistance mechanisms [43,72]. Overcoming these resistance mechanisms would potentiate the effectiveness of TRAIL in treatment of melanoma. A number of previous authors have drawn attention to the possibility of using other treatments in combination with TRAIL to overcome resistance to TRAIL-induced apoptosis [76–80]. SBHA appears to sensitize melanoma to TRAIL-induced apoptosis at multiple stages in the apoptotic pathway, and therefore may be effective in sensitizing melanoma cells to TRAIL despite the presence of different resistance mechanisms. It remains however unclear which protein is the most critical in sensitizing melanoma to TRAIL by SBHA. More detailed studies using mRNA interference assays to knock out individual proteins would

provide valuable information in this regard. It is of particular importance that co-treatment with SBHA at nontoxic doses together with TRAIL did not induce apoptosis in melanocytes. This suggests that co-administration of SBHA and TRAIL may not be toxic to normal tissues but a wider range of normal cells needs to be tested, and confirmed by *in vivo* studies. Similarly, the effectiveness of SBHA in sensitization of fresh isolates of melanoma to TRAIL-induced apoptosis needs to be examined together with studies on human xenografts to see its potential effectiveness *in vivo* [81].

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