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Invited review

Histone deacetylase inhibitors for treatment of hepatocellular carcinoma

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Key words

hepatocellular carcinoma; histone deacetylase inhibitors; cancer therapy

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; HAT, histone acetyltransferase; HDAC, histone deacetylase; NB, sodium butyrate; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; PBA, phenylbutyrate; HA-But, hyaluronic butyric ester; HA, hyaluronic acid.

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Surgical resection has been considered the optimal treatment approach, but only a small proportion of patients are suitable candidates for surgery, and the relapse rate is high. Approaches to prevent recurrence, including chemoemboliza-tion before and adjuvant therapy after surgery, have proven to have a limited benefit; liver transplantation is successful in treating limited-stage HCC because only a minority of patients qualify for transplantation. Therefore, new therapeutic strategies are urgently needed. Because in addition to the classical genetic mechanisms of deletion or inactivating point mutations, epigenetic alterations, such as hyperacetylation of the chromatin-associated histones (responsible for gene silencing), are believed to be involved in the development and progression of HCC, novel compounds endowed with a histone deacetylase (HDAC) inhibitory activity are an attractive therapeutic approach. In particular, pre-clinical results obtained using HA-But, an HDAC inhibitor in which butyric acid residues are esterified to a hyaluronic acid backbone and characterized by a high affinity for the membrane receptor CD44, indicated that this class of compounds may represent a promising approach for hepatocellular carcinoma treatment.

Introduction

Hepatocellular carcinoma (HCC) is currently the fifth most common solid tumor worldwide and the fourth most common cause of cancer-related death[1]. The incidence of HCC is approximately 1 000 000 cases worldwide; and although in Europe and the USA the incidence of HCC is 4/100 000, it is 120/100 000 in Asia and Sub-Saharan Africa[2]. In particular, statistical data from the last decade show that HCC is the second most prevalent cause of cancer deaths in men and the third most prevalent cause in women in mainland China, with more than 500 000 new cases every year[3,4]. Moreover, a rise in the incidence of disease and mortality from HCC, most likely reflecting the increased prevalence of hepatitis C virus (HCV), has recently been observed also in Japan, Western Europe and the USA[5,6].

The major etiologies of HCC are well defined, and some of the steps in its molecular pathogenesis have been elucidated. HCC arises commonly, but not exclusively, in the context of liver injury, which leads to inflammation, hepatocyte regeneration, liver matrix remodeling, fibrosis and, ultimately, cirrhosis. In fact, cirrhosis represents the most important risk factor for HCC (70%–90% of cases of HCC develop in cirrhotic liver) and it is principally imputable to chronic viral hepatitis B and/or C (HBV, HCV), although other risk factors are alcohol abuse, metabolic liver disease (such as hemochromatosis, α_1-antitrypsin deficiency and steatosis), androgenic steroid use and aflatoxin exposure[7]. In particular, chronic HBV infection is strongly associated with hepatocellular cancer in China[8], while HCV infection and subsequently cirrhosis is the leading cause of chronic hepatitis and HCC in Japan and Western countries[9].

Current trends in the management of HCC

The rates of early detection, treatment and prevention of HCC are poor, and a majority of patients (70%–85%) are affected by advanced or unresectable disease. Despite the

many treatment options, the prognosis of HCC remains dismal. In fact, even for those patients who undergo resection, the recurrence rate can be as high as 50% at 2 years[10,11] and a meta-analysis that evaluated the results of 37 randomized clinical trials of systemic and regional chemotherapy in more than 2000 HCC patients concluded that non-surgical therapies were ineffective or minimally effective[12]. In addition, most published studies of systemic chemotherapy report a response rate ranging from 0% to 25%; treatment failure is most likely due to the particular resistance to cytostatic agents displayed by HCC cells[12,13], which are known to express the multidrug-resistant gene MDR-1[14]. Liver-directed therapies, such as transarterial chemoembolization or percutaneous ethanol injection, are palliative treatments, with encouraging results only for patients with small HCC[15,16].

Alternative therapeutic approaches have been investigated, but with disappointing results. The effectiveness of hormone therapy with anti-estrogens, anti-androgens or somatostatin analogues has been studied in several trials[17,18], but these treatments were found to be generally ineffective or to not produce reproducible results[19].

At present, orthotopic liver transplantation is considered the only curative treatment option for HCC, bringing about an increase in the 5-year survival rate from the historical 20%–36% to the recent 61%, which is likely related to adoption of the Milan criteria at US transplantation centers[23,24]. However, most patients with cirrhosis are not generally considered good candidates for liver transplantation, or they remain on the waiting list until they die from tumor progression or cirrhosis-related complications.

An understanding of tumor biology and the key molecular events leading to HCC development is therefore fundamental for identifying new therapeutic strategies that are effective against HCC but not toxic to normal cells, and are well-tolerated by the typical patient with underlying cirrhosis.

HCC biology and targeted therapies

In most types of cancer, hepatocarcinogenesis is a multistep process involving different genetic alterations, including cellular oncogene activation, tumor suppressor gene inactivation (possibly in cooperation with genomic instability), DNA repair defects, overexpression of growth and angiogenic factors, and telomerase activation, which ultimately lead to malignant transformation of the hepatocyte[20]. For a more comprehensive review of the complex molecular pathogenesis of HCC the reader may refer to some excellent papers including those by Ozturk[21], Moradpour and Wands[22], Thorgeirsson and Grisham[7] and Suriawinata and Xu[23].

On the basis of knowledge of the molecular pathways that are associated with the malignant phenotype, new and promising agents, which are specifically targeted to tumor cell receptors or signaling events, are emerging. This is the case for the selective growth factor receptors, tyrosine kinase inhibitors, the fundamental role of which is to block signal transduction[24,25], or farnesyltransferase inhibitors, which counteract oncogene *ras*-mediated signaling[26]. Moreover, to stimulate an immunological response against liver tumors, other agents have been tested, for example interferon-α, which can significantly prolong the survival of HCC patients when given at high doses[27], interleukin-2, which can produce objective remission when given alone[28] or in combination with melatonin[29], or tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL), which has not yet been tested in humans, but has been found to be effective in pre-clinical experiments[30].

Gene therapy is a new and promising therapeutic strategy that is based on the introduction of genetic material, for example natural genes, chimeric genes or subgenomic molecules, into cells in order to generate a beneficial effect against disease[31]. So far, a variety of gene therapy approaches have been designed to treat liver cancer, including the replacement of functional tumor suppressor genes[32], inhibition of oncogenes[33], selective prodrug activation within the tumor[34], stimulation of antitumor immunity[35] and inhibition of tumor vascularization[36], although encouraging results have been mostly only obtained in pre-clinical models.

Histone acetylation status and HCC development

In addition to the classical genetic alterations (chromosomal deletions and rearrangements, and gene amplifications and mutations) first recognized as being responsible for hepatocarcinogenesis, molecular approaches have recently been used to identify alterations in the epigenetic control of gene transcription that positively mediate cellular proliferation or inactivate tumor suppressor genes[37]. In particular, acetylation, methylation and phosphorylation of the N-terminal lysine tails of the chromatine core histones have been found to play a critical role in post-translational modifications, suggesting the hypothesis of a histone "code"[38]. Among these modifications, the acetylation status of the histones constitutes the major epigenetic alteration, and is fundamentally involved in transcriptional regulation[39].

The acetylation level of histones is determined by the equilibrium between the activities of two groups of enzymes, histone acetyltransferases (HAT) and histone deacetylases

(HDAC), which respectively add or remove acetyl groups from the lysine tails[40]. Hypoacetylated histones are associated with a more packaged chromatin structure and with suppression of gene transcription, whereas highly acetylated histones activate gene transcription by releasing the chromatin structure[41]. At present, eleven mammalian HDAC have been identified, and these have been ordered into 3 classes[42]. Class I deacetylases (HDAC 1, 2, 3, and 8) share homology in the catalytic sites; class II includes HDAC 4, 5, 6, 7, 9, 10, and 11, of which HDAC 4, 5, 7, and 9 share homology in the C-terminal catalytic domain and N-terminal regulatory domain, whereas HDAC11 contains conserved residues in the catalytic core regions shared by both classes I and II, and HDAC6 and HDAC10 have two regions that are homologous with the class II catalytic site. The third class of HDAC is the conserved nicotinamide adenine dinucleotide-dependent Sir2 family. Increasing evidence indicates that HDAC are not redundant in function and distribution: class I HDAC are found exclusively in the nucleus, whereas class II HDAC shuttle between the nucleus and cytoplasm in response to certain cellular signals[43]. HDAC do not bind directly to DNA, but are recruited by protein complexes that can differ in their subunit composition.

The balance between HAT and HDAC activity in regulating DNA folding and gene transcription can be disrupted by HDAC inhibitors, which act by blocking HDAC enzymes; HDAC inhibition leads to lysine residue hyperacetylation and to DNA conformation changes (Figure 1). Inaccessible promoter regions thus become available targets for transcription factors, which activate the re-expression of several genes, including those involved in cell growth arrest, differentiation and apoptosis[44,45].

HDAC inhibitors and cancer treatment

Several lines of evidence suggest that inappropriate transcriptional activation commonly occurs in the formation of many types of cancer and that an imbalance between HAT and HDAC activity may be responsible for the alteration. Because HDAC dysregulation has been demonstrated in many solid cancers, including hepatocellular carcinoma, HDAC inhibitors have been investigated for their therapeutic potential to reprogram transcription and inhibit tumor cell growth and progression[46]. Historically, sodium butyrate (NB), which is normally present in the human colon as a product of the metabolic degradation of complex carbohydrates by colonic bacteria, was the first compound found to cause an increase in histone acetylation[47] and to regulate the physiological differentiation of colonocytes[48], suggesting its possible use in the prevention of colorectal cancer

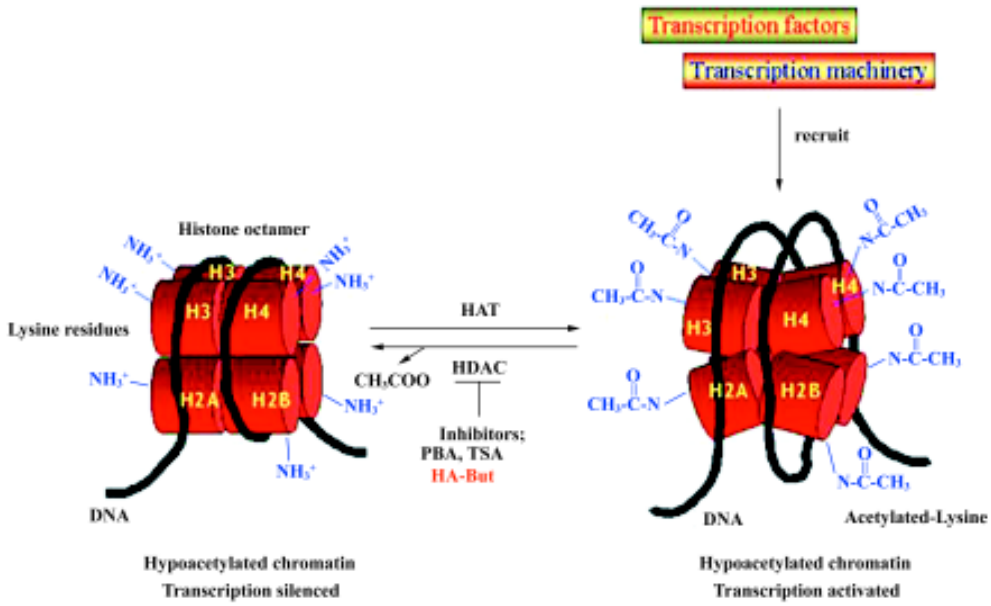

Figure 1. Schematic diagram of the activity of the histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme inhibitors, which lead to chromatin hyperacetylation or hypoacetylation, respectively. HDAC inhibitors such as phenylbutyrate (BPA), trichostatin A (TSA) and HA-But act by restoring the re-expression of several genes, including those involved in cell growth arrest, differentiation and apoptosis.

and the treatment of premalignant and neoplastic lesions. Many other HDAC inhibitors have since been identified, which belong to several chemical structure classes: 1) short-chain fatty acids, of which NB represents the prototype; 2) hydroxamic acids, including trichostatin A (TSA)[49] and a series of hydroxamic acid-based hybrid polar compounds, such as suberoylanilide hydroxamic acid (SAHA)[50]; 3) cyclic tetrapeptides, which may or may not contain the 2-amino-8-oxo-9,10 epoxy-decanoyl moiety (trapoxins A and B[51], FK228/depsipeptide[52] and apicidin[53]); and 4) benzamides (MS-275)[54] and others, such as valproic acid[55] and oxamflatin[56].

Several of these compounds, including SAHA, MS-275 and FK228/depsipeptide, are undergoing phase I and II clinical trials to examine their potential use as anticancer drugs for solid and hematological tumor treatment either as monotherapies or in combination with other cytotoxic and differentiation agents. For example, in a phase I clinical trial, SAHA was well tolerated when administered either intravenously or orally, and it was found to have antitumor activity in heavily pre-treated patients with advanced solid and hematological tumors. Furthermore, SAHA has good bioavailability when administered orally and it induces objective responses in patients with prior therapy-resistant cutaneous T cell lymphomas[57]. Similarly, a phase II trial demonstrated that FK228/depsipepide was active against refractory neoplasms[58] and chronic lymphocytic and acute myeloid leukemia[59]. MS-275 is also well-tolerated when orally administered in patients with refractory or relapsed hematological malignancies, and it is biologically active in terms of histone acetylation[60]. However, none of these studies have been specifically focused on hepatocellular carcinoma, and in addition, despite the encouraging results obtained for several types of tumors, these drugs do not achieve the major goal in cancer therapy: to selectively target anti-cancer molecules to organs or compartments that harbor tumor cells.

HA-But as an HDAC inhibitor target delivery

As mentioned earlier in this paper, NB was the first compound found to cause an increase in histone acetylation[50], so given its antiproliferative and differentiation activities, together with a relative absence of systemic toxicity, it was a candidate for the prevention of colorectal cancer and a therapeutic agent for the treatment of pre-neoplastic and neoplastic lesions. Unfortunately, the first clinical study undertaken using high doses of NB resulted in only a partial and temporary remission, principally due to the relatively low potency of the drug and a low plasma concentration that was not sufficient to inhibit cell growth, but high enough to induce side-effects[61]. Both to overcome chemical constraints that restrict the clinical application of NB, and to specifically target the compound to cancer cells, a bioconjugate (HA-But), in which a hyaluronic acid backbone was used as a suitable carrier for butyric residues, was developed[62].

Hyaluronic acid (HA), also referred to as hyaluronan, is a polysaccharide molecule whose repeated disaccharide motif comprises *D*-glucuronic acid and *D*-*N*-acetylglucosamine linked together through alternating β-1,4 and β-1,3 glycosidic bonds. HA is present in all vertebrates and is a major constituent of the extracellular matrix, where it is organized, by specific interactions, with other matrix macromolecules[63]. HA has a high rate of turnover (in the bloodstream its half-life is 2–5 min) because it is rapidly captured by receptors on hepatic sinusoidal endothelial cells, which internalize it, and subsequently catabolize it in lysosomes. Sinusoidal endothelial cells actively remove almost 90% of the circulating HA, even though the spleen is also involved in its degradation[64]. The chemical properties of HA determine its physiological role as an essential structural element in the extracellular matrix, where it regulates the retention of water molecules in the interstitial space. In addition, HA provides support for cell orientation through some specific cell surface receptors, including CD44[65]. CD44 is a single-pass transmembrane glycoprotein consisting of 4 functional domains: the distal extracellular domain (responsible for the binding of HA), the membrane-proximal extracellular domain (whose sequence depends on the alternative CD44 mRNA splicing), the transmembrane domain (similar to that of many other single-pass proteins), and the cytoplasmic domain (which has protein motifs that either interact with the cytoskeletal proteins or are responsible for intracellular signaling)[68]. Although physiologically expressed by some normal human epithelial and mesenchymal cells, where it plays an important role in immune recognition, cell-cell aggregation and cell-matrix-cell signaling, CD44 is overexpressed in most human cancers, including hepatic carcinoma, and is associated with tumor progression[66,67]. In fact, clinical evidence indicates that in comparison with normal hepatocytes, HCC is frequently associated with an increased expression of CD44 receptors[73,74], an overexpression that provides them with an essential migration-promoting advantage as demonstrated by Lara-Pezzi *et al*[68]. In their interesting pre-clinical study, these authors demonstrated a link between CD44 expression and HBV infection. In fact, a CD44-dependent migratory phenotype was induced by the stable transfection of Chang liver cells with the gene coding for the hepatitis B virus X protein (HBx), which was able to

enhance cell motility by altering the cellular morphology and inducing the formation of pseudopodal protrusions and cytoskeletal rearrangements together with the polarization of cell-surface adhesion molecule CD44. This finding is particularly relevant because it supports the relationship between HBV infection and hepatocarcinogenesis, underlining the role of HBx protein in the transformed phenotype.

In developing HA-But synthesis, we took advantage of some molecular properties of HA that satisfy some important biochemical concerns. In fact, HA can make stable bonds with butyric residues, increasing their *in vivo* half-life without affecting their pharmacological activity. In addition, we exploited its high affinity with the CD44 receptor, which is generally overexpressed on tumor cell membranes, to selectively target the butyric residues directly to neoplastic lesions, with minimal effects on normal cells. In fact, in agreement with data in the literature[69], we found that in normal cells such as fibroblasts, which express a percentage of CD44-positive cells (as evaluated by flow cytometry) similar to that of tumor cells, HA-But had no effect, suggesting that it is only really effective in actively proliferating cells, such as tumor cells (Figure 2).

HA-But: pre-clinical *in vitro* results

When we analyzed the antiproliferative effect of HA-But on two hepatocellular carcinoma cell lines, namely Hep3B and HepG2, which both expressed CD44 receptors (although to different extents), we found that after 6 days of treatment it exerted a dose-dependent effect, almost completely inhibiting the CD44-rich Hep3B (90%) cells, and moderately (but significantly) inhibiting (60%) the CD44-poor HepG2 cells; furthermore, this growth arrest corresponded to a block in the G_0/G_1 phase of the cell cycle[70]. As shown in Figure 3, the progression of the cell cycle is regulated by cyclin/cyclin-dependent kinase (cdk) complexes and cdk inhibitors, such as $p16^{ink4}$, $p21^{waf1}$, and $p27^{kip1}$ proteins. Because it is known that overexpression of cdk inhibitors leads to cell-cycle arrest and apoptosis, we investigated the effect of HA-But on the expression of some of the molecules responsible for

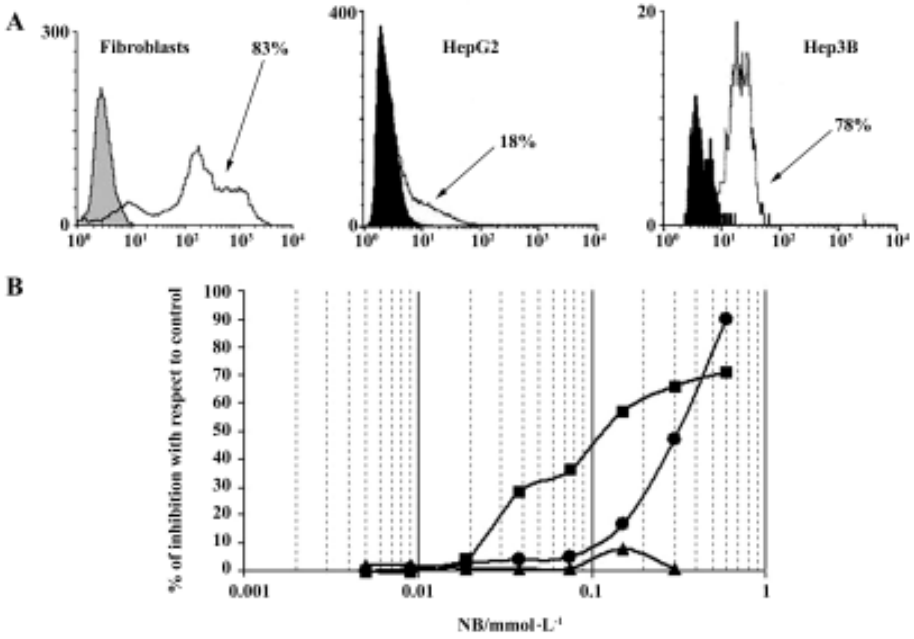

Figure 2. A: Cell surface CD44 expression on fibroblasts, and HepG2 and Hep3B cell lines as evaluated by flow cytometry. Cells (1×10^6) were first incubated with mouse antihuman CD44 antibody, and then with goat FITC-labeled antimouse secondary antibody. Negative controls (gray or black) and stained samples (white) are shown. B: Antiproliferative effect of scalar doses of HA-But on the growth of fibroblasts (▲), and HepG2 (■) and Hep3B (●) cell lines, after 6 d of treatment. Briefly, cells (1000 cells per well) were seeded in 96-well plates in RPMI-1640 medium supplemented with 10% fetal calf serum and allowed to adhere for 24 h. The seeding medium was removed and replaced with experimental medium supplemented with increasing concentrations of HA-But. At the end of the experiments the antiproliferative effect was evaluated by using the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method. Results are expressed as percentage of inhibition with respect to control (cells maintained in the presence of culture medium alone). Each point represents the mean value from 3 independent experiments. The variation coefficient was <5% and therefore error bars are not shown.

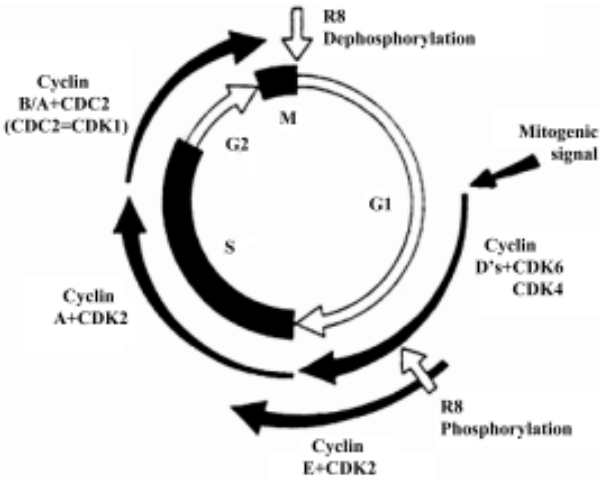

Figure 3. Schematic representation of cell cycle phases. In proliferating cells, the cell cycle consists of 4 phases. Gap1 (G1) is the interval between mitosis and DNA replication, which occurs during the synthesis (S) phase. The S phase is followed by a second gap phase (G2), during which growth and preparation for cell division occurs. Mitosis and the production of 2 daughter cells occur in M phase. Passage through the 4 phases of the cell cycle is regulated by a family of cyclins that act as regulatory subunits for cyclin-dependent kinases (cdks). The activity of the various cyclin/cdk complexes that regulate the progression through the G1-S-G2 phases of the cell cycle is controlled by the synthesis of the appropriate cyclins during a specific phase of the cell cycle. In addition, there are several proteins, including $p16^{ink4}$, $p21^{waf1}$, and $p27^{kip1}$ (termed cdk inhibitors) that can inhibit the cell cycle in G1 when an adverse event, such as DNA damage, has occurred.

growth arrest, including cyclin D1, which is involved in the regulation of the G_1 phase of the cell cycle, and whose gene is found amplified in 10%–20% of HCC,[71] but leads, when combined for example with $p16^{ink4}$ amplification, to a loss of growth control in more than 30% of HCC. Experimental findings indicated that as in other types of tumors, such as lung cancer[65], and in agreement with the effect of other HDAC inhibitors on hepatoma cells[72,73], HA-But increased cdk-inhibitor expression (ie, the protein level of $p27^{kip1}$ and $p21^{waf1}$), while decreasing cyclin D1 protein levels, suggesting that the HA backbone does not interfere with the activity of butyric residues, which maintain their biological properties. In fact, as previously demonstrated in lung cancer[65], and similarly to NB, HA-But induces a hyperacetylation of histone H4, a dose-dependent overexpression of $p27^{kip1}$ and $p21^{waf1}$, and a block of cell growth in the G_0/G_1 phase of the cell cycle. In addition, cytometric analysis showed that CD44 receptor turnover was not affected by treatment with HA-But, which is a finding of great pharmacological relevance, because the stable presence of the receptors on plasma membranes guarantees a continuous internalization of the drug.

cDNA microarray analysis is a technical approach that enables investigators to measure the expression of thousands of mRNAs simultaneously in a biological specimen, providing comprehensive information that is useful for diagnosis and therapeutic intervention. Data gathered using this technique have further confirmed the previous experimental findings regarding the mechanism of action of HDAC inhibitors, and demonstrated that almost all HDAC inhibitors exerted their antiproliferative effects by modulating a small set of genes that regulate key cellular activities such as proliferation and differentiation. In particular, it has been shown that TSA, SAHA, depsipeptide are able to modulate genes involved in apoptosis and cell cycle pathways, among which are cdk inhibitors such as $p16^{ink4}$, $p21^{waf1}$, and bcl-2[74,75]. In addition, gene expression profiling of hepatocellular carcinomas has provided qualitative and quantitative evidence that the genes involved in the development and progression of a HCC correlate with the dysregulation of pathways associated with cell cycle regulation[76], apoptosis[77], signal transduction[78], cellular adhesion and angiogenesis[79,80]. Thus, the finding that HDAC inhibitors, including HA-But, can restore growth control and induce differentiation is particularly exciting.

HA-But: pre-clinical *in vivo* results

There are very few studies that have aimed to investigate the *in vivo* effect of HDAC inhibitors on tumor growth or the metastatic spread of liver tumors. Among the studies that have been conducted, a study using 4-phenylbutyrate, a derivative of the short-chain fatty acid butyrate, found that when administered via intratumor catheter, the compound reduced the growth of xenograft tumors derived from hepatocarcinoma cell lines, most likely by the induction of $p21^{waf1}$ expression and the activation of apoptosis[78].

The *in vivo* capability of HA-But to inhibit primary tumor growth and metastatic spread has been investigated in several animal models[65,75], and evaluation of the drug biodistribution, by using the compound labeled with ^{99m}Technetium, found that a few minutes after iv administration, there was a substantial accumulation of the compound in the liver, uniformly distributed in both lobes[75]. These results have been confirmed by the evaluation of the *ex vivo* distribution of HA-But, which showed that the liver was the organ of preferential accumulation, in agreement both with the finding obtained by using native HA[81] and with the observation that circulating hyaluronan is physiologically degraded by hepatic sinusoidal endothelial cells

via CD44 receptors[82]. All these findings clearly suggest that iv injection can be appropriately exploited to treat intrahepatic lesions. In fact, when we explored the therapeutic potential of HA-But for the treatment of intrahepatic lesions induced in the mouse by intrasplenically inoculated Lewis lung carcinoma cells (LL3) and B16/F10 melanoma cells (two cell lines known for their particular aggressiveness[83,84], and which express high percentages of CD44-positive cells), we found that a prolonged iv administration of HA-But affected the survival time of tumor-bearing animals, reducing the number of intrahepatic metastatic lesions. In addition, prolonged treatment with low doses of HA-But significantly increased the survival time of treated mice relative to untreated controls. It is noteworthy that 90 d after tumor implantation, 80% of HA-But treated animals were still alive versus approximately one-third in the untreated group[75].

Conclusions and future perspectives

Experimental evidence and preliminary clinical phase I and II trials indicate that HDAC inhibitors acting on a pivotal mechanism of gene transcription such as histone acetylation may represent an innovative therapeutic approach for solid and hematological cancer. In particular, recent findings indicate that in pre-clinical studies HA-But is able to inhibit hepatocellular carcinoma cell growth, and that this antiproliferative activity is due to an increase in some cell-cycle related proteins, such as $p21^{waf1}$ and $p27^{kip1}$, and a decrease in some others, including cyclin D1. Furthermore, the results provide experimental evidence for the clinical use of HA-But as a promising agent for the treatment of hepatocellular carcinoma, a tumor that is otherwise particularly resistant to chemotherapy. The treatment exploits the overexpression of CD44 receptors on tumor cell membranes, which allows selective targeting of the compound to the neoplastic lesion.

In addition to DNA acetylation, which hampers gene transcription, aberrant DNA hypermethylation of cytosine residues in the promoter region can cause growth-controlling gene silencing, a frequently observed phenomenon for several genes in HCC[7]. Therefore, the additional inhibition of DNA methyltransferase (DNMT), the enzyme responsible for such hypermethylation (the activity of which can be reversed using specific DNMT inhibitors, such as 5-aza-2-deoxycytidine, 5-AZA) could be an interesting candidate for further study. Therefore, a promising development for HA-But could be a new chemical compound in which HA-But is simultaneously esterified with 5-AZA. Such a molecule should be able to reactivate silenced genes and enhance the re-expression of specific genes involved in cell growth arrest, terminal differentiation and apoptosis in aggressive tumors such as hepatocellular carcinoma.

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