

Hyaluronan: a suitable carrier for an histone deacetylase inhibitor in the treatment of human solid tumors

Review Article

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Abbreviations: butyric acid (But); clusters of differentiation, (CD); extra-cellular matrix receptor type III, (ECMRIII); hyaluronic acid (HA); hyaluronic butyric ester (HA-But); histone deacetylase (HDAC); hyaladherin, (Hyal); Lewis lung carcinoma, (LL3); liver endothelial cell clearance receptor, (LEC receptor); receptor for hyaluronate-mediated motility, (RHAMM); suberoylanilide hydroxamic acid, (SAHA); trichostatin A, (TSA)

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Summary

Histone deacetylase (HDAC) inhibitors are an exciting new class of drugs potentially useful as anti-cancer agents. These compounds, most of which have been identified during the last decade, can induce growth arrest, terminal differentiation and/or apoptosis in a variety of solid and haematological tumors. This review focuses on a newly synthesized cell-targeted bioconjugate (HA-But) obtained by esterification of butyric acid (But), the smallest HDAC inhibitor, with hyaluronic acid (HA), a natural linear polymer, consisting of repeating disaccharidic units, which selectively recognizes CD44, a transmembrane receptor over-expressed in most malignant tumors. *In vitro*, HA-But has provided to be 10-fold more effective than But in inhibiting cells proliferation of a panel of human cancer cell lines, representative of the most common human cancers and, similarly to But, has been shown to regulate the expression of some cell cycle-related proteins, to induce growth arrest in the G₁/G₀ phase of the cell cycle and to increase hystone acetylation indicating that the presence of the hyaluronan backbone does not interfere with the biological activity of butyric residues. *In vivo*, pharmacokinetics studies have shown different rate of distribution of radiolabeled HA-But according to the route of administration (i.v., i.p. or s.c.): few minutes after i.v. treatment, with substantial accumulation in the liver and spleen; relatively slow rate after i.p. or s.c. treatment, with a marked persistence of the drug in the site of injection in the case of s.c. administration. In addition, in mice HA-But treatment has demonstrated a marked efficacy in inhibiting primary tumor growth and lung metastases formation from Lewis lung carcinoma (LL3) and in inhibiting liver metastases originating from intra-splenic implant of LL3 or B16-F10 melanoma cells. In particular, the effect of s.c. and i.p. treatment with HA-But on liver metastases resulted respectively in 87% and 100% metastases-free animals, and in a significant prolongation of the survival time compared to the control groups. Present findings suggest a possible clinical application of this hyaluronic butyric ester as antiproliferative agent in primary and metastatic lesions.

I. Introduction

Hyaluronan is one of the main constituent of extracellular matrix where it is organized by specific interactions with other matrix macromolecules and provides an essential support for cells orientation through the binding with specific cell surface receptors, the most important of which is CD44. CD44 is involved in a variety of cellular functions including leukocyte rolling, cell-cell

aggregation, matrix-cell and cell-matrix signalling, receptor-mediated internalization and cell migration. In addition to these physiological properties, CD44 has been found to be overexpressed in most tumor cells and associated with tumor progression.

Aim of this review is to describe the *in vitro* and *in vivo* results obtained using a novel bioconjugate in which hyaluronan is used as a carrier for butyric acid, the

smallest inhibitor of histone deacetylases (a class of enzyme involved in the regulation of gene expression), known to induce growth arrest, terminal differentiation and/or apoptosis in a variety of solid and haematological tumors.

II. Hyaluronan: structure and chemical-biological properties

In 1934 Karl Meyer and his assistant John Palmer isolated and described a novel glycosaminoglycan from the vitreous of bovine eyes that they named hyaluronic acid (HA) (Meyer and Palmer, 1934). This was the birth for one of the nature's most versatile and fascinating macromolecules. Today this macromolecule is most frequently referred to as hyaluronan reflecting the fact that it exists *in vivo* as a polyanion and not in the acid form. However, it would take an additional 20 years before Meyer's laboratory completed the experimental work with the definition of the precise chemical structure of the basic disaccharide motif that forms hyaluronan (Weissman and Meyer, 1954): D-glucuronic acid and D-N-acetylglucosamine linked together through alternating -1,4 and -1,3 glycosidic bonds (**Figure 1**).

In the cell, hyaluronan synthase enzymes (Has1, Has2 and Has3), localized in the plasmatic membrane, synthesize this linear, high molecular weight polymer in which the number of repeating disaccharide units can reach 10,000 or more and a molecular mass of about 4 million of Daltons (each disaccharide unit is about 400 Da).

Hyaluronan 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. In some cases, including vitreous of the human eye, synovial fluid or in the matrix produced by the cells around the oocyte prior to ovulation, hyaluronan is important in giving to this fluids their visco-elastic properties; in others, including hyaline cartilages and connective tissues, it serves as an essential structural element in the matrix, regulating the water molecules retention in the interstitial space, and providing a support for cell orientation (Tammi et al, 2001).

Hyaluronan has an extraordinarily high rate of turnover in vertebrate tissues. A 70-kilo individual has 15 g of hyaluronan, 5 g of which turns over daily, and in the bloodstream, hyaluronan has a half-life of two to five minutes. In fact, a large proportion of the hyaluronan molecules are rapidly captured by receptors on hepatic sinusoidal endothelial cells, which internalize them for subsequent catabolism in lysosomes. Sinusoidal endothelial cells actively remove almost 90 percent of the circulating hyaluronan, even though the spleen is also involved in its degradation (Fraser et al, 1985). Degradation is predominantly enzymatic (mainly by hyaladherins) and occurs in a highly controlled stepwise fashion with a discrete decrease in polymer size which results in a series of hyaluronan fragments having widely different biological activities depending on chain lengths. In fact, high and low molecular weight chains appear to have opposite effects on cell behaviour. While the extracellular high molecular weight (about 10^7 Da) chains are space-filling molecules that hydrate tissues, appear in the earliest stages of wound healing, when spaces must be created to facilitate polymorphonuclear leukocytes infiltration of the wound area, bind fibrinogen (Weigel et al, 1986; Frost and Weigel, 1990), inhibit angiogenesis (Feinberg and Beebe, 1983), and are anti-inflammatory and immune-suppressive (McBride and Bard, 1979; Delmage et al, 1986), small hyaluronan fragments, in the 6-20 kDa size range, interact with a different set of receptors that trigger signalling cascades, are highly angiogenic (promote differentiation of the endothelial cells) and potent stimulators of inflammatory cytokines and of dendritic cells (Termeer et al, 2000, 2003; Noble, 2002).

III. CD44 receptor

To organize themselves, cells interact with extracellular matrix through specific cell surface receptors recognizing hyaluronan, several of which have been identified. They include: CD44, Receptor for Hyaluronate-Mediated Motility (RHAMM), LYVE, HARE and Liver Endothelial Cell clearance receptor (LEC receptor)

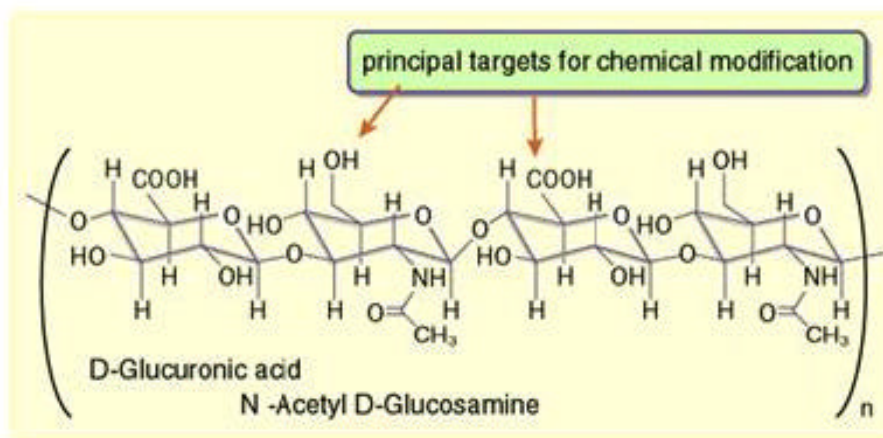


Figure 1. Hyaluronan basic structure. The repeating disaccharide unit consists of --4) -D-glucuronic acid (1->3) -D-N-acetylglucosamine-(1--

(Entwistle et al, 1996; Pilarski et al, 1999; Day and Prestwich, 2001), but this list is likely to increase in the future. Nevertheless, the major hyaluronan receptor and the most studied to date is CD44 (Isacke and Yarwood, 2002). CD44 is a plasma membrane-associated multistructural and multifunctional glycoprotein, which derives its name from its identity with a family of common leukocyte antigens (Clusters of Differentiation, CD) as defined by the International Workshop on Human Leucocyte Differentiation Antigens. However, prior to its designation as CD44, other names had been given to this protein, including Pgp-1, Hermes antigen, HUTCH-1, HCAM, ECMRIII (Extra-Cellular Matrix Receptor type III) as well as the common functional name of hyaluronan receptor.

CD44 identification, obtained in the late 1970s, derived from the observation that in many studies on cell-cell aggregation hyaluronan was found to mediate cross-bridging adjacent cells suggesting the existence of membrane-localized hyaluronan binding sites.

CD44 is a single-pass transmembrane glycoprotein of approximately 85 kD consisting of four functional domains (**Figure 2A**). The *distal extracellular domain* is the region primarily responsible for the binding of hyaluronan; the *membrane-proximal extracellular domain* is the primary site of alternative splicing of CD44 mRNA responsible for the different isoforms of CD44 and is the site of insertion for lateral glycosaminoglycan chains; the *transmembrane domain* is similar to that of many other single-pass protein; the *cytoplasmic domain* or tail, which

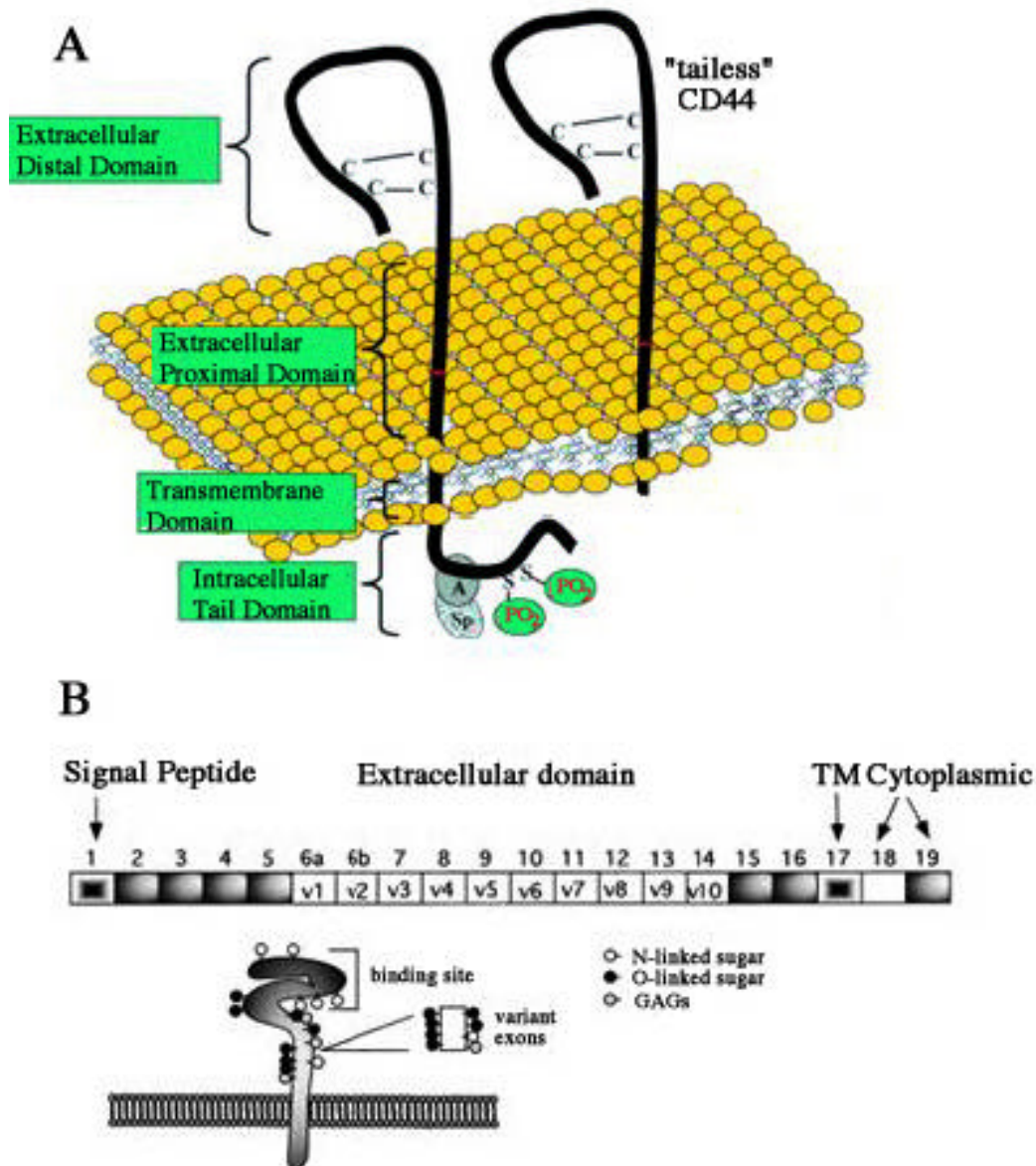


Figure 2. (a) Schematic CD44 receptor structure. The *distal extracellular domain* is the region primarily responsible for the binding of hyaluronan; the *membrane-proximal extracellular domain* is the primary site of alternative splicing of CD44 mRNA responsible for the different isoforms of CD44 and is the site of insertion for lateral glycosaminoglycan chains; the *transmembrane domain* is similar to that of many other single-pass protein; the *cytoplasmic domain* or tail, which exhibits protein motifs responsible for the interaction with cytoskeletal proteins and intracellular signalling. (b) alternative splicing of CD44 exons. The exons that encompass the CD44 gene are numbered and diagrammed. V1-v10 are the variant exons that exhibit extensive rearrangements due to alternative splicing resulting in a myriad of variant isoforms of CD44.

exhibits protein motifs responsible for the interaction with cytoskeletal proteins and intracellular signalling. As other transmembrane glycoprotein receptors, CD44 undergoes extensive post-translational modifications including phosphorylation, N- or O-glycosylation and addition, in some CD44 isoforms, of glycosaminoglycan chains. All these modifications are being actively explored as potential modulators of hyaluronan binding or of other CD44 functions. The CD44 gene, located on chromosome 11p13, consists of 20 exons (**Figure 2B**) and encodes for multiple mRNA transcripts, which arise from the alternative splicing of 12 of the 20 exons (Isacke and Yarwood, 2002). The standard and most prevalent form, termed CD44s, consists of a protein encoded by exons 1-5, 16-18, and 20 and exhibits an *extracellular domain* (exons 1-5 and 16), a highly conserved *transmembrane domain* (exon 18), and a *cytoplasmic domain* (exon 20) that selectively interacts with cytoskeletal proteins and regulates specific signalling.

Alternative splicing of exons 6-15, also named variant exons v1-v10, originates several CD44 isoforms with an increased length of extracellular domain ranging in size from 80 to 250 kDa. In addition, either exon 19 or 20 may be differentially expressed because of alternative splicing and generate two variations of the intracellular "tail" portion of the molecule. The alternatively spliced message, containing exon 19 instead of exon 20, generates a short-tailed form of the CD44 protein, lacking of intracellular signalling motifs and of protein domains necessary for interaction with cytoskeletal components. Since the inhibition of the expression of short-tailed form induces an increase in hyaluronan internalization, it has been postulated this isoform as a dominant negative, with a modulatory function on the protein/cytoskeletal interaction.

Little information is available concerning the regulation of CD44 gene expression. CD44 is transcriptionally upregulated by proinflammatory cytokines, such as interleukine 1 which increases CD44 mRNA and protein expression levels in chondrocytes and vascular smooth muscle cells, or by growth factors such as epidermal growth factor and transforming growth factor- β , which upregulate CD44 expression in fibroblasts, in several tumor cell types, and in epithelial cells undergoing stratification.

CD44 is known to participate in a wide variety of cellular functions, including leukocyte rolling on hyaluronan (Clark et al, 1996), cell-cell aggregation, matrix-cell and cell-matrix signalling, receptor-mediated internalization/degradation of hyaluronan and cell migration. With regard to hyaluronan internalization, as shown in **Figure 3A**, a minimum of six hyaluronan sugar residues, corresponding to three repeating disaccharidic units, are requested for the binding to the cell surface via CD44. The high molecular weight extracellular polymer is constrained to the cell surface by the combined efforts of CD44 and the GPI-anchored enzyme hyaladherin (Hyal)-2 located in specialized invaginations of the plasma membranes (caveolae) composed of cholesterol and gangliosides, termed lipid rafts, significant because they also recruit a large number of key signalling molecules. As

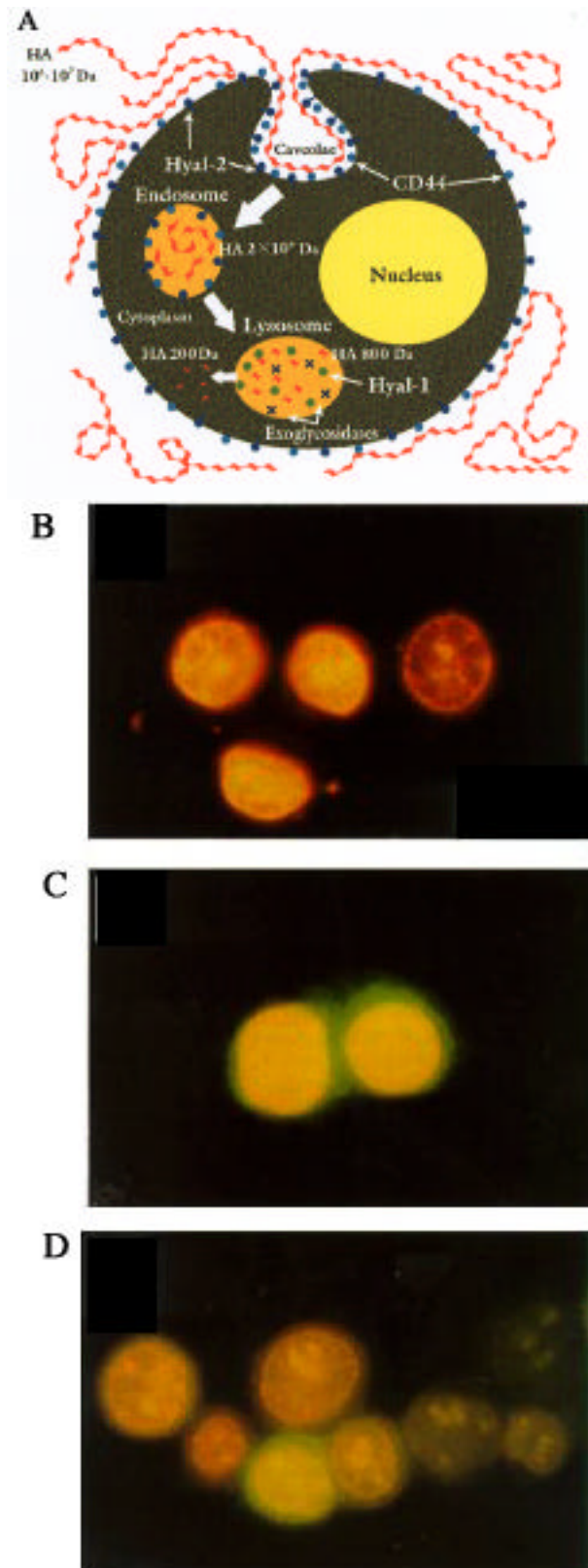


Figure 3. (a) CD44-mediated hyaluronan internalization. Hyaluronan sugar residues, corresponding to three repeating disaccharidic units, bound to the cell surface via CD44, is internalized by invagination of the plasma membrane and degraded by lysosome. (b-d) CD44-mediated HA-But internalization. (b) negative control; (c) after 4 h of treatment with fluoresceinated HA-But; (d) after 4 h of treatment with fluoresceinated HA-But plus monoclonal antibody anti-CD44 (Reproduced from Coradini et al, 1999 with kind permission from International Journal of Cancer).

demonstrated by autoradiography using ^3H -labeled hyaluronan (Hua et al, 1993), the caveolae then becomes an endosome that subsequently fuses with a lysosome completing the degradation of the hyaluronan. The hyaluronan polymer is then cleaved to 20 kDa limit fragments (Lepperdinger et al, 1998) corresponding to about 50 disaccharide units, around the size at which a stable tertiary structures is expected to form (Scott and Heatley, 2002). These Hyal-2-generated hyaluronan fragments are further degraded by Hyal-1 to small disaccharides that when sufficiently small can diffuse out of lysosomes into the cytoplasmic compartment or alternatively may leave the lysosome through specific transporters, as other metabolites do, such as amino acids and other sugars. This process is quite rapid as we have observed incubating cells expressing unoccupied CD44 receptors with fluorescein-tagged hyaluronan (**Figure 3B-D**) and it is specifically CD44-mediated as demonstrated by the block of fluorescein-tagged hyaluronan internalization in the presence of an anti-CD44 antibody (Coradini et al, 1999). The specificity of hyaluronan/CD44 interaction has been also demonstrated using fluorescein-labeled dextran, a polysaccharide with structure and molecular weight similar to hyaluronan, but unable to bind CD44. Experiments performed under similar conditions, clearly demonstrated a lack of dextran internalization, supporting the hypothesis that hyaluronan internalization does not occur via simple fluid phase pinocytosis but rather via a CD44-mediated active process.

IV. CD44/hyaluronan interactions and tumor invasion and metastasis

In the last years there has been intense interest in the association of CD44 expression and tumor progression and metastasis. It is not surprising that many malignant cell types overexpress CD44 because it is expressed by many cells prior to their cancer transformation. However, although CD44 is expressed by some normal human epithelial and mesenchymal cells, differential screening for epitopes present on tumor cells revealed that cancer phenotype is more likely associated with alternatively spliced isoforms of CD44 and in particular with CD44v6 variant. This isoform contains an inserted amino acid sequence within the *extracellular domain* of the molecule which derives from the addition of exon 11. Interest in CD44 variants peaked when it was found that transfection of nonmetastatic tumor cells with CD44v6 isoform enhanced cell efficiency for metastasis (Gunthert et al, 1991; Seiter et al, 1993) and that conversely it was inhibited by the addition of an anti-CD44 monoclonal antibody (Guo et al, 1994; Breyer et al, 2000) or an antisense oligonucleotide against CD44 (Reeder et al, 1998; Harada et al, 2001). From these initial observations, numerous studies have documented the prevalence of CD44 variant isoforms in human cancers, including the expression of alternatively spliced combinations of the v3, v6 and v9 isoforms (Knudson, 1998). Since most tumors, particularly solid cancers, are often enriched in hyaluronan, which provides them an essential migration-promoting micro-environment, enhanced expression of

CD44 may represent an efficient way to facilitate locomotion through the tumor-associated hyaluronan-rich matrix.

Preclinical studies have demonstrated a correlation among cellular capacity for CD44-mediated endocytosis, hyaluronan degradation and tumor metastatic aggressiveness: the malignant cells that better internalize and degrade hyaluronan appear to be the most metastatic (Zuhalka et al, 1995; Strobel et al, 1997). These experimental findings have been confirmed also in clinical studies conducted on different type of tumors. Studies on patients with non-Hodgkin's lymphoma have demonstrated a direct relation between tumor progression and CD44v6 isoform expression, suggesting this variant as an independent prognostic factor (Stauder et al, 1995). Similar observations have been reported also for colorectal (Wielenga et al, 1993; Mulder et al, 1994), gastric (Mayer et al, 1993), pancreatic (Gunthert et al, 1991), renal (Terpe et al, 1996), hepatocellular (Endo and Terada, 2000), cervical (Kainz et al, 1995), ovarian (Uhl-Steidl et al, 1995), non-small lung (Hirata et al, 1998), breast carcinoma (Kaufmann et al, 1995; Martin et al, 1997), and melanoma (Manten-Horst et al, 1995), in which the presence of the isoforms, and in particular of CD44v6, was associated with advanced stage, unfavourable clinicopathological features and an adverse prognosis. Taken together these findings indicate that CD44 variants expression represents an important new acquisition for the knowledge of tumor cell and assumes a prognostic value in systemic as well as in solid tumors.

V. Histone deacetylase inhibitors

One of the most important aspects of the complex network that controls normal cell functions is the regulation of gene expression through chromatin structure rearrangement, which depends on the level of acetylation of the chromatin-associated histones and regulates the access of transcription factors to DNA (Grunstein, 1997). The degree of histones acetylation depends on a dynamic equilibrium between two classes of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC), which respectively add or remove acetyl groups from the amino-terminal lysine residues of histones. Eleven mammalian HDACs have been till now identified and ordered into three classes. Class I deacetylases (HDACs 1, 2, 3 and 8) share homology in the catalytic sites; class II includes HDACs 4, 5, 7, and 9 which share homology in C-terminal catalytic domain and the N-terminal regulatory domain, whereas HDAC11 contains conserved residues in the catalytic core regions shared by both class I and II, and HDAC6 and HDAC10 have two regions of homology with the class II catalytic site. The third class of HDACs is the conserved nicotinamide adenine dinucleotide-dependent Sir2 family of deacetylase (De Ruijter et al, 2003).

There is increasing evidence that HDACs are not redundant in function and distribution (Khojbin et al, 2001). Class I HDACs are found exclusively in the nucleus, whereas class II shuttle between the nucleus and cytoplasm upon certain cellular signals (De Ruijter et al, 2003). HDACs do not bind directly to DNA but are

recruited by protein complexes that may differ in their subunit composition.

Balance between HAT and HDACs activity can be disrupted by HDAC inhibitors, whose mechanism of action is based on their ability to inhibit the activity of HDAC enzymes. Inhibition of HDAC activity, thus enhances HAT activity leading to histones hyperacetylation and DNA unfolding; nonaccessible promoter regions become thus available targets for transcription factors, allowing the re-expression of several genes, including those responsible for cell growth control and differentiation (van Lint et al, 1996). In fact, experimental evidences indicate that HDAC inhibitors induce growth arrest, differentiation and/or apoptotic cell death of a broad spectrum of transformed cells including both haematological cancers and solid tumors (Marks et al, 2001a). HDAC inhibitors represents, therefore, a new class of potentially effective anticancer agents (Marks et al, 2001b).

The HDAC inhibitors so far identified belong to several chemical structural classes including: (a) *short-chain fatty acid*, of which sodium butyrate (the sodium salt of butyric acid) is a prototype (Candido et al, 1978); (b) *hydroxamic acid*, including trichostatin A (TSA) (Yoshida et al, 1990) and a series of hydroxamic acid-based hybrid polar compounds, such as suberoylanilide hydroxamic acid (SAHA) (Richon et al, 1998); (c) *cyclic tetrapeptides* containing or not the 2-amino-8-oxo-9,10 epoxy-decanoyl moiety (Depsipeptide, Apicidin) (Darkin-Rattray et al, 1996; Nakajima et al, 1998); (d) *benzamides* (MS 27-275) (Saito et al, 1999). Class I and II, but not class III HDACs are inhibited by all these compounds through a direct interaction between the inhibitor and the enzyme as demonstrated by crystallographic analysis (Finnin et al, 1999).

In the next paragraphs we will focus on the smallest of them: butyric acid.

VI. Butyric acid and butyric derivatives

Butyric acid (But) is a short-chain fatty acid naturally present in the human colon in millimolar concentrations and is produced by colonic bacteria during the metabolic degradation of complex carbohydrates (Cunning, 1981; Hill et al, 1995; Pryde et al, 2002). *In vivo* experiments have demonstrated an increasing gradient of But concentration from the crypts to the lumen, that plays a pivotal role during the normal turnover of mucosal epithelium (Roediger, 1980; McIntyre et al, 1993). In fact, low But concentrations induce physiological colonocyte proliferation and migration from colon crypts towards the lumen, where higher concentrations induce cell growth arrest, differentiation and, finally, apoptosis (Whiteley et al, 1996). A decrease in physiological concentrations has been proved to be associated to the alterations in cell growth and differentiation observed in the adenoma-carcinoma sequence (Clausen, 1995).

According to its mechanism of action as an histone deacetylase inhibitor, But modulates the activity of several transcription factors, including AP1, c-myc and Sp1, through which it regulates the expression of many proteins

that control cell proliferation (Tabuchi et al, 2002). In particular, we have observed that But is able to modulate some cell cycle-related proteins including cyclin D1, p53, p27^{kip1} and p21^{waf1} (Coradini et al, 2000; Pellizzaro et al, 2001), this latter supposed to be one of the genes most commonly induced by HDAC inhibitors; its expression has been found to directly correlate with histones acetylation (Richon et al, 2000).

As a consequence of this regulatory activity, But increases differentiation markers expression, such as alkaline phosphatase in colon cancer cells (Coradini et al, 2000) or induces apoptosis probably through the activation of caspase 3 and 7 expression (Hague, 1995).

Due to its antiproliferative and differentiating activities, associated with the relative absence of systemic toxicity, But has been proposed for the prevention of colorectal cancer (Scheppach et al, 1995) and as therapeutic agent for the treatment of preneoplastic and neoplastic lesions (Sowa and Sakai, 2000). However, the first clinical study undertaken using high doses of sodium butyrate (Miller et al, 1987) resulted only in a partial and temporary remission from disease, principally due to the relative low potency of the agent (to be effective the drug must be administered at millimolar concentrations), to the low effective plasma concentrations, which are insufficient to inhibit cell growth, but sufficient to induce side effects including hypernatremia, and to the rapid clearance (t_{1/2}=6 minutes) which resulted in a very short half-life requiring a continuous administration in order to obtain suitable plasma concentrations (Daniels et al, 1998).

However, in consideration of the antiproliferative and differentiating effects of But, supported by many preclinical studies, new chemical derivatives have been developed to overcome the constraints which hampered But clinical application. To obtain compounds able to increase But *in vivo* effectiveness over a sustained period while satisfying the important requirements for specificity and low toxicity, different approaches have been investigated, most of them implying the association with a chemically suitable drug delivery, able to stabilize the molecule and reduce its degradation. Thus, Pouillart et al, (1991) have synthesized monosaccharidic derivatives, which were hampered by the rapid metabolism of monosaccharides in the organism (Planchon et al, 1992). They also reported the synthesis of triglyceride derivatives in which one or two butyric residues were covalently bound to glycerol substrate, containing one or two palmitic acid molecules (Planchon et al, 1993). Another group developed a pro-drug based on the acyloxyalkyl derivatives of carboxylic acids, which release butyric acid after intracellular hydrolysis (Rephaeli et al, 1991) and one of them, AN9 (pivaloyloxymethyl butyrate), has been shown to be effective in inducing cell growth inhibition at a concentration 10-fold lower than But (Aviram et al, 1994; Zimrah et al, 1997). In addition, phenol butyric derivatives, such as phenylbutyrate have been investigated, but clinical trials studies seem to indicate a limited drug efficacy associated with central nervous system toxicity (Gore et al, 2002).

To overcome both chemical and pharmacological constraints we decided to take advantage of hyaluronan

properties and to use it as a carrier on which covalently bind many butyric residues simultaneously in order to increase the drug/carrier ratio (**Figure 1**). Choosing hyaluronan as a carrier for the delivery of butyric acid, some important chemical concerns have been taken into account: 1. the bond between the carrier and the drug must be sufficiently stable to increase the *in vivo* half-life of the active principle without effect on its efficacy; 2. the carrier must be a molecule that the organism will not eliminate too rapidly and must be devoid of toxic side effects. In addition, hyaluronan responds to a major challenge in cancer therapy: the selective delivery of an anti-cancer molecule of small size directly into the tumor cells through the linking to a cellular receptor of the water-soluble, polymer-drug conjugates (Lou and Prestwich, 2002). Following this approach, to increase the therapeutic potential of But by a selective targeting and contemporarily decrease its undesirable side effects, we have developed a new bioconjugate constituted by the smallest HDAC inhibitor covalently linked to hyaluronan, which in addition to an high chemical versatility and a good biocompatibility, is characterized by the selective uptake to CD44, overexpressed in most human cancers.

Moreover, a not negligible effect of such a conjugate could be the disruption, by the presence of exogenous hyaluronan, of the interaction between CD44 receptors, expressed on tumor cell membranes, and the hyaluronan of the extracellular matrix. Since CD44-hyaluronan interaction is an important requirement to promote tumor growth and metastasis spread, the administration of exogenous hyaluronan which competes for CD44 binding could markedly reduce local growth and dissemination. This detrimental effect on tumor growth and spread could be mediated also through the inhibition of neoangiogenesis, the formation of new blood vessels from existing vascular network, which allows a tumor mass to overcome the constraints related to the lack of the oxygen and nutrients required for growth and spread (Carmeliet and Jain, 2000). Through a complex mechanism of action, which implies also the synthesis of some angiogenesis-related factors, the most important of which is the Vascular Endothelial Growth Factor (VEGF), tumor cells activate proliferation and migration of endothelial cells (Siemeister et al, 1998) that respond to the angiogenic stimulus overexpressing CD44 on their plasma membrane and moving through the extracellular matrix towards the tumor mass to be vascularized (Trochov et al, 1996). Since we have demonstrate that But is able to modulated VEGF synthesis (Pellizzaro et al, 2002), the new bioconjugate may act also as an antiangiogenic agent.

In the next paragraphs we will describe the most important preclinical results obtained with such an interesting derivative.

VII. *In vitro* studies with the hyaluronic butyric derivative

Hyaluronic butyric ester (HA-But) has been synthesized as described in Coradini et al, (1999) starting from HA with a molecular weight of about 85 kDa and the procedure has been further refined to improve synthesis control (Coradini et al, 2004a). Briefly, the esterification

reaction is accomplished by means of an activated form of butyric acid, i.e., an adduct between butyric anhydride and 4-dimethylaminopyridine. Using this synthetic strategy a better control of the reaction, in terms of yield and conversion, is performed and a butyric-ester derivative with a degree of substitution (i.e., the ratio between the number of butyric residues and the number of repeating disaccharidic unit of the polysaccharide) ranging from 0.1 to 0.8 is obtained.

It is interesting to note that, when evaluated as a function of the degree of substitution, the growth inhibitory activity of HA-But has been found inversely related to the number of butyric residues bound per disaccharidic unit with an high Spearman correlation coefficient ($r_s=0.984$, $P=0.01$) which suggests that the presence of too many butyric residues could hamper the binding of HA to CD44 receptor, due to the shielding of the functional groups involved in the recognition process. The *in vitro* growth inhibitory activity of HA-But, in comparison to But, has been evaluated on a large series of cancer cell lines, representative of the most widely diffuse human solid tumors: breast (MCF7 and MDA-MB231), ovary (IGROV1), prostate (DU145), bladder (253J), colon (HT29), liver (HepB3 and HepG2), pancreas (MiaPaCa), lung carcinoma (NCI-H460 and NCI-H460M) and melanoma (JR8). All responded to the antiproliferative effect of HA-But with a dose-dependent inhibition of cell growth higher than that of But. The higher effectiveness of HA-But with respect to But alone is evident considering the ratio between the effectiveness of the drug (expressed in term of the concentration which inhibits cell growth by 50% of the control, IC_{50}) and that of But alone (**Table 1**). In fact, in all but one cell lines the ratio $IC_{50}HA-But/IC_{50}But$ has been found below 1 and in some of them (i.e., DU145, HT29, NCI-H460, NCI-H460M, HepB3 and HepG2) the drug have shown an antiproliferative activity 7-30 fold higher than that of But. These results suggest that the use of HA as a carrier for butyric acid can significantly improve its biological activity without any chemical alteration for But or cytotoxic effect due to the presence of HA backbone, that alone produces an almost null cytotoxic effect (Coradini et al, 2004a, 2004b). The finding that the inhibitory activity is obtained in all the tumor cells investigated, despite their different histological origin, is not surprising taking into account the pivotal role of a HDAC inhibitor on gene expression during cell replication, as confirmed by the lack of any effect on normal slowly proliferating fibroblasts, notwithstanding the high expression of CD44 (83%) (Coradini et al, 2004a). It is interesting to note that the metastatic subclone of non-small lung carcinoma (NCI-H460M) responds to HA-But to an extent similar to that of the parental clone (NCI-H460) probably because of the similar rate of growth (expressed as time of duplication) and expression of CD44 receptors. This finding is of particular relevance since it supports the possibility of using HA-But also for the treatment of metastatic lesions as successfully assessed in *in vivo* animal models and described in paragraph VIII.

Cytometric analysis showed that, as expected (Knudson, 1998), all tumor cell lines overexpressed CD44

receptors on their plasma membrane, even though in a different extent (from 18% to 97%) and that the receptor turnover is not affected by the treatment with HA-But. This last finding is of particular pharmacological relevance, since the constant presence of the receptors on plasma membrane guarantees a continuous internalization of the drug. However, our data indicate that the quantitative expression of CD44 does not appear tightly correlated to its biological effect. In fact, despite the presence of CD44 receptors is a fundamental requirement for the effectiveness of HA-But, no relationship was found between the extent of CD44 expression and the inhibitory activity of the drug (**Table 2**): in fact a similar antiproliferative effect is observed in CD44-poor and in CD44-rich cell lines. For example, HepG2 and NCI-H460, two cell lines characterized by a different CD44 expression (18% and 91%, respectively), respond to HA-But in a similar manner suggesting that, after a sufficiently prolonged treatment interval (in this case 6 days), the drug is effective also in CD44-poor tumor cells, probably due to the very rapid CD44 turnover which guarantees a constant presence of the receptor and therefore a continuous internalization. Anyway, even though CD44 is the major receptor for hyaluronan, other membrane receptors including RHAMM, could be responsible for such an internalization (see paragraph III).

With regard to the mechanism of action, HA-But exerts an effect very similar to But indicating a lack of interference of HA backbone in the activity of butyric

Table 1. Effect of HA-But, with respect to But expressed in terms of IC₅₀ values

Cell line	But IC ₅₀ (mM) mean±SD	HA-But IC ₅₀ (mM) mean±SD	HA-But IC₅₀ But IC₅₀
MCF7	0.6±0.030	0.2±0.012	0.33
MDA-MB231	0.3±0.015	0.4±0.023	1.33
IGROV1	0.6±0.033	0.4±0.025	0.67
DU145	1.0±0.06	0.033±0.002	0.03
253J	0.8±0.043	0.4±0.020	0.50
HT29	1.4±0.069	0.2±0.010	0.14
HepB3	3.0±0.134	0.31±0.026	0.19
HepG2	2.1±0.116	0.12±0.052	0.17
MiaPaCa	1.8±0.09	0.7±0.04	0.39
NCI-H460	1.3±0.068	0.12±0.005	0.08
NCI-H460M	0.8±0.041	0.1±0.006	0.13
JR8	0.8±0.044	0.4±0.022	0.50

Cell lines were kept for 6 days, an interval time sufficient to observe a statistically significant difference with respect to control, in the suitable medium supplemented with increasing concentrations of HA-But (range: 0.001 - 4 mg/ml) or sodium butyrate (range: 0.001 - 4 mM). Experiments were performed at least twice and samples were run in eight replicates. At the end of the experiments the antiproliferative effect was evaluated using MTT or Alamar Blue method, for adherent or floating cells, respectively. IC₅₀ was defined as the concentration of drug that inhibits cell growth by 50% of the control. Ratios between IC₅₀ values for the compounds significantly different from 1 (equal IC₅₀ for the two compounds) are bolded.

Table 2. Relationship between CD44 expression and inhibitory effect of HA-But in a representative series of cell lines

Cell line	CD44 expression (%)	HA-But IC ₅₀ (mM)
HepG2	18	0.12
MCF7	60	0.20
HT29	71	0.20
HepB3	78	0.31
NCI-H460	91	0.12
NCI-H460M	91	0.10
JR8	97	0.40

The expression of CD44 receptors was evaluated by flow cytometry using a murine monoclonal antibody raised against human CD44 (clone 5F12, Neo Markers). Parallel fresh samples (1x10⁶ cells) were incubated first with primary antibody at a dilution of 1:20 for 60 min at room temperature and then with a secondary FITC-conjugated goat anti-mouse antibody (Sigma) at a dilution of 1:50 for 30 min at room temperature in the dark. The negative control sample was incubated with the secondary antibody alone. The fluorescence of stained cells was measured using a FACScan flow cytometer. The fluorescence signal was collected in linear and logarithmic mode; at least 30,000 events were recorded for each sample.

residues, which maintain their biological properties. In fact, HA-But induces an hyperacetylation of histone H4, a dose-dependent overexpression of some G₁/S transition-related proteins, including the cyclin-dependent kinase inhibitors p27^{kip1} and p21^{waf1}, and the block of cell growth in the G₀/G₁ phase of cell cycle (Coradini et al, 2004a).

VIII. *In vivo* studies with the butyric derivative

The *in vivo* capability of HA-But to inhibit primary tumor growth and metastatic spread has been investigated in several animal models. However, in a preliminary series of experiments, pharmacokinetics and toxicity studies have been performed to investigate the HA-But biodistribution according to the different routes of administration and the possible side effects. Therefore, for pharmacokinetics purposes, HA-But has been labelled to technetium-99m (^{99m}Tc), the γ -emitting radioisotope most widely used in radiodiagnosis and as a radioactive probe in pharmacological studies (Vittori et al, 1997). An efficient labelling method allowed us to directly anchor ^{99m}Tc to HA polymer with minor changes in charge, conformation and hydrophilicity and no significant changes in biodistribution and physiological interactions (Jurisson, 2002) but obtaining labelling yields of about 95%. Thus, solutions containing ^{99m}Tc-HA-But have been administered i.v., i.p. or s.c. to healthy male CBA/Lac mice and scintigraphic images have been collected with a 5-min interval for 1 hr after i.v. injection, with a 30-min interval for 2 h after i.p. administration and with a 10-min interval for 6 h after s.c. injection, using a YAP camera, a γ -camera with an high spatial resolution, specifically

designed for the imaging analysis of the *in vivo* distribution of radiolabelled compounds evaluated by a dedicated software (Giron, 2002).

The results have indicated that, with regards to i.v. injection, few minutes after treatment, there is a substantial accumulation of the compound in liver, uniformly distributed in both lobes (Figure 4) which becomes more intense after 1 hour. Scintigraphic images indicate that the compound accumulates also in the kidneys probably in relation to excretion of the fragments produced by the polymer degradation. The results have been confirmed by the evaluation of *ex-vivo* distribution of

HA-But which shows the liver as the organ of preferential accumulation in agreement with the finding obtained with native HA (Gustafsson et al, 1994) and the observation that circulating hyaluronan is physiologically degraded by hepatic sinusoidal endothelial cells via CD44 receptor (Seagusa et al, 2002). In addition, imaging analysis indicated an accumulation of ^{99m}Tc-HA-But also in the spleen as expected considering the role of the spleen in HA degradation (Laurent et al, 1995). In fact, considering the amount of labeled HA-But accumulated in a given organ and expressed as percentage of the injected dose (%ID) liver and spleen accumulate respectively 45% and

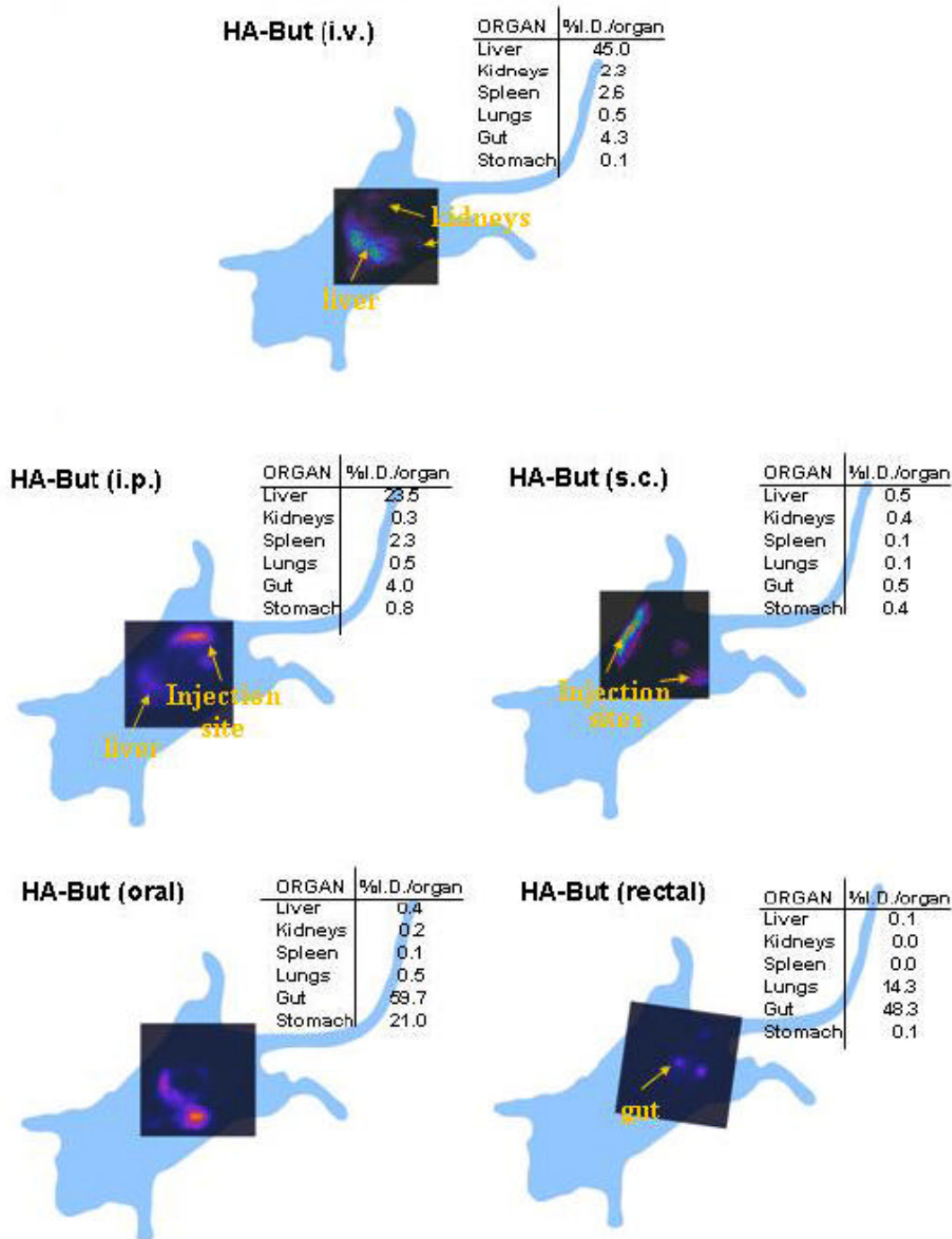


Figure 4. *In vivo* distribution of ^{99m}Tc HA-But was investigated using a YAP camera, a high spatial resolution γ -camera. Scintigraphic images of mouse abdomens were obtained 1 h after i.v. or oral, 2h after i.p., 6 h after s.c. administration of an HA-But saline solution containing ^{99m}Tc-HA-But. Arrows indicate the main sites of accumulation.

2.6% of the injected dose. However, when considering the amount of ^{99m}Tc -HA-But accumulated per g of tissue (expressed as percentage of the injected dose per gram of tissue, % I.D./g) and thus taking into account the size of each organ, the spleen accumulated 11.66% I.D./g, an amount consistent with that found in the liver, which accumulated 15.83% I.D./g.

Liver uptake decreases considerably when the labelled compound is administered intraperitoneally or subcutaneously. In fact, i.p. (**Figure 4**) administration reduces the accumulation in the liver to a 23.5% which further decreases to a 0.5% after s.c. administration (**Figure 4**). Interestingly, scintigraphic images collected 6 hours after s.c. administration showed that a 36% of injected ^{99m}Tc -HA-But is still localized at the site of injection. These differences in HA-But pharmacokinetics, depending on the route of administration, may be exploited to appropriately target HA-But: i.v. route could be used for treating intrahepatic lesions, whereas s.c. route may be more useful for treating local lesions or to partially bypass the hepatic drug segregation.

To obtain a complete information on HA-But biodistribution, a series of experiments have been performed also administering ^{99m}Tc -HA-But orally or rectally. As shown in **Figure 4**, oral administration results in a partial retention of the drug in the stomach (21% I.D.) and in an accumulation in the duodenum (59.7% I.D.). Scintigraphic images collected after 1 hour following rectal administration indicated that the drug localizes preferentially in the colon (48.3% I.D.) with a remarkable retention also in the rectum (14.3% I.D.). *Ex-vivo* analysis has clearly demonstrated that ^{99m}Tc -HA-But was actively internalized by the colonocytes suggesting a topic use of HA-But for treating colorectal carcinomas.

Acute and subacute toxicity experiments have indicated that HA-But administered i.v., i.p., or s.c. and followed up for 30 days did not cause toxicity. In particular, as reported in **Table 3**, when administered i.v., HA-But LD₅₀ (i.e., the dose which induces lethal effect in a 50% of the animals) is higher than 0.4 mg/ml, which was the maximum injectable dose permitted by the solution viscosity. When administered i.p. or s.c., HA-But LD₅₀ values are respectively superior to 0.1 mg/ml and 0.2 mg/ml. During a 30-day follow up interval no animals died. Subacute toxicity experiments, performed administering i.p. 0.1 mg/ml HA-But for 10 days consecutively or injecting s.c. 0.04 mg/ml for 25 days consecutively, indicate a complete lack of toxicity

confirmed by the observation that during the 90-day follow up interval no animals died.

On the basis of the *in vitro* results, pharmacokinetics and toxicologic indications and keeping with the main sites of localization for human solid tumors, three major applications of HA-But have been explored: intratumor administration for treating localized lesions, such melanoma, i.v. injection for treating intrahepatic lesions and s.c. administration for treating lung metastases. In fact, liver and lung, in addition to harboring primary tumors, are often targets for metastatic spread from primaries arising in other organs, including colorectal carcinoma, breast cancer and melanoma.

Therefore, to investigate the *in vivo* pharmacological activity of HA-But we used three murine experimental models: a. subcutaneously inoculated mammary tumor cells (MCa), able to induce both local and lung lesions; b. subcutaneously inoculated LL3, able to induce both local and lung lesions; c. intrasplenic inoculated LL3 or melanoma cells (B16/F10), both able to induce intrahepatic lesions. In addition, we have explored the activity of HA-But also in a systemic model: the intraperitoneally inoculated lymphoma cells (TLX5), able to induce both intraperitoneal ascitis and brain metastases.

For the evaluation of the HA-But effect in treating localized tumor lesion, female mouse s.c. inoculated with MCa cells were treated intratumorally 11 days after cells inoculum with 0.05 mg/ml/day for 9 days. As shown in **Figure 5**, the intratumor treatment with a free of toxicity dose of HA-But significantly reduced primary tumor size as compared to untreated controls with a statistically significant difference starting from day 20. Moreover, intratumor injection of HA-But also reduced the number (-51%, $P < 0.05$) and the weight (-51%, $P < 0.05$) of lung metastases produced by MCa with a statistically significant difference in comparison to the untreated animal group.

Similar results have been obtained when the effect of HA-But was investigated in the s.c. inoculated LL3 cells model, able to induce local and lung metastatic lesions, and intratumorally treated with HA-But (0.05 mg/ml/day) for 9 days starting from day 12. Also in this case, intratumor injection of HA-But reduced (-70%, $P < 0.01$) primary lesion size and decreased the number (-45%, $P < 0.05$) and the weight (-65%, $P < 0.01$) of lung metastases with a statistically significant difference in comparison to the untreated animal group (Coradini, 2004a).

Table 3. Acute and subacute toxicity of HA-But after i.p., s.c. and i.v. administration

	Dose (mg/ml/mouse)	Route of administration	Mortality death/total
<u>Acute</u>	0.1	i.p.	0/5
	0.2	s.c.	0/5
	0.2	i.v.	0/5
	0.4		0/5
<u>Subacute</u>	0.1	i.p.(x10 days)	0/10
	0.04	s.c.(x25 days)	0/15

The observation time was of 30 days and the survival time has been evaluated at 90 days.

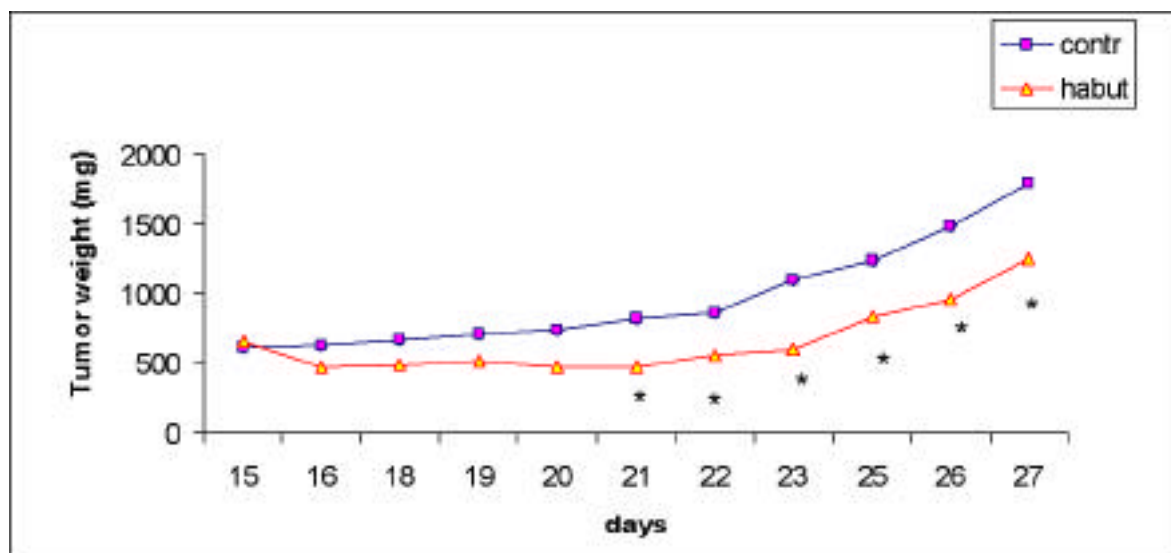


Figure 5. *In vivo* effect of intratumor administration of HA-But in CBA/Lac female mice (10 per group) inoculated s.c. with 1.5×10^6 MCa cells at day 0 and treated from day 11 for 9 days with HABut $6 \mu\text{m}/\text{mouse}$. Primary tumor growth was evaluated every day and lung metastases were evaluated at day 27 at the sacrifice of the animals.

Even though we have no direct information on the effect of HA-But on cell motility and/or invasive potential of the metastatic cell line used in our experiments it is likely to assume that the compound should be able to compete for the binding to CD44 receptors with the endogenous components of the extracellular matrix (Skubitz, 2002) and to exert a detrimental effect on cell motility and therefore on invasion potential (Alaniz et al, 2002). The most exciting results have been obtained treating with HA-But the intrahepatic lesions induced by the intrasplenically inoculated LL3 or B16/F10 cells, two cell lines known for their particular aggressiveness (Barbara-Guillem et al, 1989; Carrascal et al, 2003) and which express high percentage of CD44-positive cells (respectively 68% and 87%). Intrasplenic inoculum model was chosen to better reproduce the biological outcome of liver metastases avoiding the use of conventional *in vivo* experimental models which imply the production of “artificial” liver colonization via intravenously injected tumor cells (Asao et al, 1992).

As shown in **Table 4**, s.c. or i.p. administration of HA-But dramatically reduced the formation of liver metastases produced by both cell lines. In particular, as regard LL3 cells, 86% of the s.c. treated animals and 87.5% of the i.p. treated animals were free of macroscopically detectable metastases, and only one animal per treatment group (i.p. or s.c.) presented metastatic foci at sacrifice (i.e., 15 days after implantation). Conversely, in the untreated group, only 14% of the s.c. treated animals and 12.5% of the i.p. treated animals were metastastase free. A similar response rate was observed also in mice that were intrasplenically implanted with B16/F10 melanoma cells; at sacrifice all s.c. or i.p. HA-But treated animals were free of macroscopically detectable liver metastases versus none of animals in both (s.c. and i.p.) control groups. In addition, histological analysis of the liver parenchyma indicated that

HA-But did not affect liver morphology (Coradini et al, 2004b).

These interesting results have been further strengthened by a parallel series of experiments in which the effect of HA-But on survival time of tumor-bearing animals was investigated. Prolonged treatment with low doses of Ha-But (s.c. 0.04 mg/ml/day plus i.p. 0.1 mg/ml on day 4,11,18,25 and 31) significantly increased ($P < 0.03$) the survival time of treated mice over untreated controls. Noteworthy, 90 days after tumor implantation 80% HA-But treated animals were still alive versus 27% in the untreated group (Coradini et al, 2004b).

With regard to the activity of HA-But on the intraperitoneally inoculated lymphoma cells (TLX5) model able to induce both intraperitoneal ascitis and brain metastases, in a preliminary experiment we have observed that the s.c. treatment with HA-But at the doses of 0.05 or 0.1 mg/ml for 7 days resulted in a dose-dependent reduction of the number of tumor cells present in the peritoneal ascitis (32% and 69%, respectively) with respect to controls (**Table 5**) not paralleled by a concomitant increase of survival time, because the animals death in this model is due to brain metastases and Ha-But cannot pass the blood-brain barrier.

IX. Conclusion and perspectives

The studies summarized in this review provide evidence that HA-But, a new bioconjugate constituted by a backbone of HA, one of the main component of the extracellular matrix, partially esterified with butyric acid, the smallest HDAC inhibitor, is a potent inhibitor of cell growth *in vitro* and an antiproliferative/antimetastatic agent *in vivo* and that hyaluronan is a very suitable carrier due to its high biocompatibility, its ability to stabilize But molecule, to specifically target But to tumor cells, to internalize it via CD44 without interfere with its mechanism of action.

Table 4. Effect of 7-day i.p. or s.c. treatment with HA-But on liver metastasis formation following intrasplenic implantation of LL3 or B16/F10 melanoma cells

Route of administration	Treatment group	LL3-induced		B16-F10-induced	
		No. of liver metastases	No. of metastasis-free animals	No. of liver metastases	No. of metastases-free animals
s.c.	control	>10	1/7	>10	0/7
	HA-But	< 5	6/7*	-	6/6*
i.p.	control	>10	1/8	>10	0/7
	HA-But	< 5	7/8*	-	7/7*

*P<0.05, with respect to control (Fisher's exact test)

Table 5. Effect of HA-But treatment in mouse lymphoma model

Treatment (mg/ml/mouse)	Survival time (days)	Peritoneal ascitis (No. of cells x 10 ⁶)
Control group	9.6±0.2	689.0±126.0
HA-But (0.05)	10.4±0.8	471.3±48.1*
HA-But (0.1)	11.6±0.6	216.3±48.5*

*P<0.05, with respect to control

Groups of 10 CBA/Lac male mice inoculated i.p. with 100.000 cells TLX5 at day 0, treated i.p. starting 24 h after tumor implant, with HA-But for 7 days. Survival time was evaluated in 5 mice, whereas other 5 mice were sacrificed at day 8 for the count of peritoneal tumor cells.

Undoubtedly, several other HDAC inhibitors, including TSA, SAHA, depsipeptide, MS-275 inhibit tumor growth in animal models and are in phase I and II clinical trials either as monotherapy or in combination with other cytotoxics and differentiation agents (Kelly et al, 2002).

For example, phase I trial with SAHA, administered either intravenously or orally, have found that the drug is well-tolerated and has anti-tumor activity in heavily pre-treated patients with advanced solid and haematologic tumors (Kelly et al, 2003). Moreover, administered orally has good bioavailability and induces responses in patients with prior therapy-resistant cutaneous T cell lymphomas. Similarly, in a phase I trial, depsipeptide has been found active against refractory neoplasms (Sandor et al, 2002) and MS-275 has been found, when orally administered, well tolerated and biological active in terms of histone acetylation (Gojo et al, 2002).

However, the use of these drugs does not allow the achievement of the major goal in cancer therapy: to selectively target anticancer molecules to organs or compartments harboring tumor cells. Conversely, HA-But which has high affinity for CD44, a specific membrane receptor provided to be overexpressed in most human cancers, including breast, colon, lung and hepatic carcinoma, could be a promising antineoplastic agent for the specific treatment of primary as well as metastatic tumors. Exploiting the overexpression of CD44 receptors on tumor cells membrane, hyaluronan is thus able to selectively target But to the neoplastic lesions. Although CD44 is expressed by some normal human epithelial and mesenchymal cells in which plays important roles in immune recognition, lymphocyte trafficking and cell-cell and cell-matrix interactions, we have demonstrated, in agreement with literature data (Byrd, 1999), that in normal cells like fibroblasts HA-But has no activity suggesting that the drug is really effective only in actively proliferating cells like tumor cells.

During the past few years the search for small molecules able to form pharmacological useful interactions with proteins that play a pivotal role in the regulation of cancer cell growth has gained an increasingly position within the drug discover process. Focusing on cancer, the discovery of small molecule compounds (such as butyric acid) able to interact with particular transcriptional targets that participate in the development and progression of cancer represents an exciting future perspective aimed in substituting the classic cytotoxic and hormonal anticancer agents with more selective drugs with greater efficacy and minimal side effects. In this light the development of drugs such as HA-But could respond to the these new strategies.

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