Hyaluronic acid butyric esters in cancer therapy

Annalisa Speranza[a], Cinzia Pellizzaro[a] and Danila Coradini[a]

In this review we focus on a promising novel histone deacetylase (HDAC) inhibitor (HA-But) obtained by the esterification of butyric acid (BA), the smallest HDAC inhibitor, with hyaluronic acid (HA), the main constituent of the extracellular matrix which selectively recognizes a transmembrane receptor (CD44) overexpressed in most primary cancers and associated with tumor progression. *In vitro*, HA-But has proved to be 10-fold more effective than BA in inhibiting the proliferation of a panel of human cancer cell lines, representative of the most common human cancers, and, similar to BA, to regulate the expression of some cell cycle-related proteins, to induce growth arrest in the G_1/G_0 phase of the cell cycle and to increase histone acetylation. *In vivo*, HA-But treatment has demonstrated a marked potency in inhibiting primary tumor growth and lung metastases formation from murine Lewis lung carcinoma (LL3) as well as liver metastases formation from intrasplenic implantation of LL3 or B16-F10 murine 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. The results suggest that the presence of the HA backbone does not interfere with the biological activity of butyric residues and that HA-But could represent a promising cell-targetable antineoplastic agent for the treatment of primary and metastatic tumors. *Anti-Cancer Drugs* 16:373–379 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, **16**:373–379

Keywords: butyrate, hyaluronic ester, histone deacetylase

[a]Unit of Biomolecular Determinants in Prognosis and Therapy, Experimental Department, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy.

Sponsorship: This work was partially supported by a grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), Rome, Italy.

Correspondence to D. Coradini, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy.
Tel: +39 02 23903053; fax: +39 02 23903052;
e-mail: danila.coradini@istitutotumori.mi.it

Received 30 November 2004 **Accepted** 27 December 2004

Introduction

One of the most important aspects of the complex mechanism that controls normal cell functions is the epigenetic regulation of gene expression, and growing evidence suggests a relationship between epigenetic modification of chromatin structure and tumor development. In fact, through the regulation of the level of acetylation of the chromatin-associated histones, the access of transcription factors to gene promoter regions can be modulated [1]. The degree of histone 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 N-terminal lysine residues of histones.

The balance between HAT and HDAC 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 enhances HAT activity, and leads to histone hyperacetylation and DNA unfolding; inaccessible promoter regions become available targets for transcription factors allowing the re-expression of several genes, including some involved in cell growth arrest, differentiation and apoptosis also in a broad spectrum of cell lines derived from solid and hematological cancers [2,3]. In addition, experimental evidence indicates that *in vivo* the antiproliferative effect of HDAC inhibitors is paralleled by an increased survival in animal models.

For these reasons, in recent years HDAC inhibitors have gained increasing attention and several molecules, belonging to different chemical classes, have been proposed as potentially effective anticancer agents [4]. Among them there are (i) short-chain and aromatic fatty acids, such as butyric acid (BA) and phenylbutyrate, (ii) hydroxamic acids, including trichostatin A (TSA) and a series of hydroxamic acid-based hybrid polar compounds, such as suberoylanilide hydroxamic acid (SAHA), (iii) cyclic tetrapeptides containing or not the 2-amino-8-oxo-9,10 epoxy-decanoyl moiety (trapoxins A and B, depsipeptide, and apicidin), and (iv) benzamides (MS 27-275).

In this review we focus on a novel class of HDAC inhibitors derived from the esterification of BA, the smallest HDAC inhibitor, with hyaluronic acid (HA), the main constituent of extracellular matrix (ECM) used as a suitable drug delivery [5,6]. We illustrate its pharmacological properties and preliminary clinical results, and discuss its potential clinical use and some interesting future perspectives.

0959-4973

Rationale for development of hyaluronic butyric esters

BA is naturally present in the human colon as a product of the metabolic degradation of complex carbohydrates by colonic bacteria and plays a pivotal role in the physiological turnover of mucosal epithelium, regulating, in a concentration-dependent manner, colonocyte proliferation, maturation and migration from the crypt to the lumen [7]. Experimental evidence indicated that a reduction in the physiological concentration of BA is associated with the alterations in cell turnover and differentiation occurring during the adenoma–carcinoma transformation [8].

According to the mechanism of action of HDAC inhibitors, BA modulates the activity of several transcription factors, including AP1 and Sp1, through which it regulates the expression of many proteins that control cell proliferation [9]. In particular, we have observed that BA is able to modulate some cell cycle-related proteins, including cyclin D1, p53, $p27^{kip1}$ and $p21^{waf1}$ [10,11], this latter supposed to be one of the most common HDAC inhibitor-induced genes. As a consequence of this regulatory activity, BA increases differentiation marker expression, such as alkaline phosphatase in colon cancer cells [10], or it induces apoptosis, probably through the activation of caspase-3 [12].

Because of these antiproliferative and differentiation activities, associated with the relative absence of systemic toxicity, BA has been proposed for the prevention of colorectal cancer, and as a therapeutic agent for the treatment of pre-neoplastic and neoplastic lesions. Unfortunately, the first clinical study undertaken using high doses of sodium butyrate (the sodium salt of BA) resulted only in a partial and temporary remission from disease [13], principally due to its relative low potency (the drug must be administered at millimolar concentrations to be effective), to its low plasma concentration, which is not sufficient to inhibit cell growth, but high enough to induce side-effects, including hypernatremia, and to its rapid clearance ($t_{1/2} = 6$ min) which resulted in a very short half-life requiring continuous administration to obtain suitable plasma concentrations.

To overcome the constraints which hamper BA clinical application (i.e. to obtain compounds able to increase BA *in vivo* effectiveness over a sustained period while satisfying the important requirements for specificity and low toxicity), new chemical derivatives have been developed, all exploiting the association of BA with a chemically suitable drug delivery, able to stabilize the molecule and reduce its rapid degradation. Among the compounds developed there are monosaccharide derivatives [14], triglyceride derivatives, in which one or two butyrate residues were covalently bound to glycerol substrate [15], and acyloxyalkyl derivatives of carboxylic acids (such as pivaloyloxymethyl butyrate), which release BA after intracellular hydrolysis [16]. However, none of these drugs responds to the main requirement of cancer therapy: to selectively target anticancer molecules to organs or compartments harboring tumor cells.

To specifically target the drug and in the meanwhile to overcome chemical constraints, we have taken advantage of the properties of HA and we have used it as a carrier to which to covalently bind many butyric residues simultaneously. With regard to drug delivery, HA satisfies some important chemical concerns: (i) it forms a stable link with BA which increases the BA *in vivo* half-life without affecting its efficacy and (ii) being the main constituent of the ECM, HA is a biocompatible molecule devoid of toxic side-effects. In addition, HA allows selective delivery of BA to tumor cells. In fact, it specifically binds to some cell surface receptors, the most important of which is CD44, a single-pass transmembrane glycoprotein that in its cytoplasmic domain exhibits protein motifs interacting with the cytoskeleton and involved in intracellular signaling. Although CD44 is expressed by some normal human epithelial and mesenchymal cells, allowing several physiological cellular functions, including cell–cell aggregation, and matrix–cell and cell–matrix signaling, it is overexpressed in most tumor cells and associated with tumor progression [17,18]. In solid tumors, enhanced expression of CD44 represents an efficient way to facilitate cancer cell locomotion through the HA-rich ECM.

Therefore, we developed a novel bioconjugate (HA-But) constituted by BA covalently linked to an HA backbone (molecular weight of about 85 kDa) [6]. Using a synthetic strategy which allows good control of the reaction it was possible to obtain HA-But derivatives with a degree of substitution (i.e. the ratio between the number of substituted hydroxyl groups and the number of repeating disaccharide units of the polysaccharide) ranging from 0.1 to 0.8. Interestingly, when we evaluated the growth-inhibitory activity of HA-But derivatives as a function of their degree of substitution, we found an inverse relationship between the number of butyric residues bound per HA disaccharide unit and the antiproliferative effect [6], which suggested that the presence of too many butyric residues could hamper the binding of HA to the CD44 receptor due to shielding of the functional groups involved in the recognition process, and induced us to continue the experiments using the less-substituted derivative only.

Cellular pharmacology

Cytotoxicity and effect on histone H4 acetylation and on cell cycle-related proteins

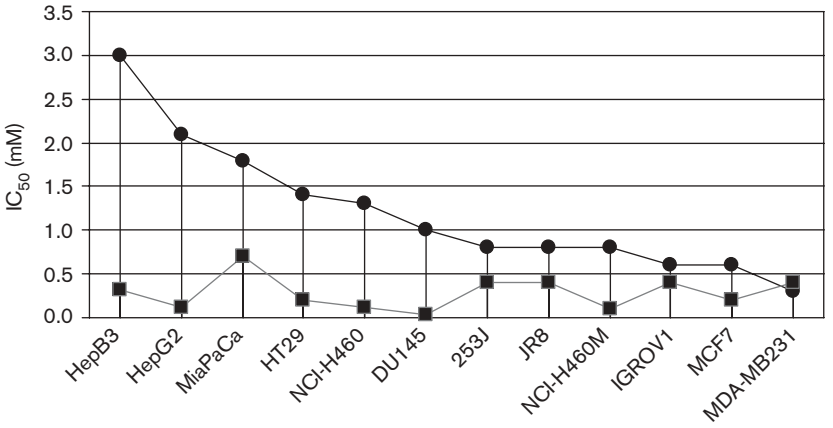
The *in vitro* growth inhibitory activity of HA-But was evaluated on a large panel of cancer cell lines,

representative of the most widely common human solid tumors: breast (MCF7 and MDA-MB231), ovary (IGROV1), prostate (DU145), bladder (253J), colon (HT29), liver (HepB3 and HepG2), pancreas (MiaPaCa), lung (NCI-H460 and its metastatic subclone NCI-H460 M) carcinoma and melanoma (JR8), all over-expressing CD44 receptor (evaluated by flow cytometry). The findings indicated that the use of HA as a carrier did not influence the biological properties of BA and significantly improved its antiproliferative activity. All but one of the tested cell lines were responsive to the antiproliferative effect of HA-But in a dose-dependent manner with a cell growth inhibition higher than that observed in the presence of BA alone. In fact, as shown in Figure 1, all cell lines, except MDA-MB231, showed an HA-But IC_{50} value (i.e. the concentration of the drug able to inhibit cell growth by 50% with respect to the control) lower than that of BA and in some of them (i.e. HT29, NCI-H460 M, HepB3, NCI-H460, HepG2 and DU145) HA-But showed an antiproliferative potency from 7- to 30-fold higher than that of BA [6,19]. The finding that HA-But was effective in all the tumor cells investigated, despite their different histological origin, is not surprising taking into account the pivotal role of an HDAC inhibitor on gene expression, this latter particularly activated in highly proliferating cells, as confirmed by the lack of any effect on normal slowly proliferating fibroblasts, used in our experiments as a paradigm of normal well-differentiated and slowly proliferating cells, notwithstanding their physiologically high CD44 expression (83% of cells were positive by flow cytometric analysis) [6].

It is interesting to note that the metastatic subclone of the non-small lung carcinoma cell line (NCI-H460 M) responded to HA-But to an extent similar to that of the parental clone (NCI-H460), probably because of the similar rate of growth and expression of CD44 receptors. In fact, in both cell lines the duplication time is about of 24 h and they overexpress CD44 receptor in a similar manner (91% of cells, by flow cytometry). This finding is of particular relevance since it supports the possibility of also using HA-But for the treatment of metastatic lesions as successfully assessed in *in vivo* animal models [6]. In addition, cytometric analysis showed that CD44 receptor turnover was not affected by the treatment with HA-But, a finding of particular pharmacological relevance, since the constant presence of the receptors on the cell plasma membrane may guarantee a continuous internalization of the drug even though the quantitative expression of CD44 seemed not to be correlated to HA-But potency. In fact, a similar antiproliferative effect was observed in CD44-poor and CD44-rich cell lines. For example, HepG2 and NCI-H460, two cell lines characterized by quite different CD44 expression (18 and 91% of cells, respectively), responded to HA-But in a similar manner, suggesting that, after a sufficiently prolonged treatment interval (in this case 6 days), HA-But was effective also in CD44-poor tumor cells, probably due to the very rapid CD44 turnover which guarantees a constant presence of the receptor in the cell membrane and therefore a continuous internalization.

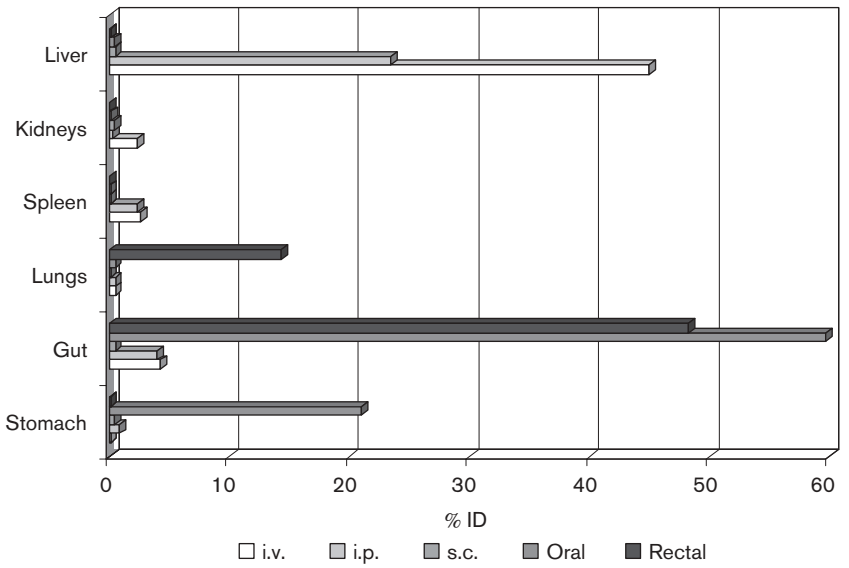
With regard to the mechanism of action, HA-But showed a biological effect very similar to BA, indicating a lack of interference of the HA acid backbone in the activity of butyric residues, which maintain their molecular properties. In fact, like BA, HA-But induced hyperacetylation of histone H4, a dose-dependent overexpression of some G_1/S transition-related proteins, including the

Fig. 1



Effect of HA-But (squares) or BA (circles) expressed as IC_{50} value. Cell lines, each maintained in appropriate culture medium, were treated with scalar concentrations of HA-But (range 0.001–4 mg/ml) or BA (range 0.001–4 mM) for 6 days, an interval of time sufficient to observe a statistically significant difference with respect to control. At the end of the experiments the antiproliferative effect was evaluated using MTT. IC_{50} was defined as the concentration of drug that inhibited cell growth by 50% of the control.

Fig. 2



In vivo distribution of [^{99m}Tc]HA-But according to the route of administration (i.v., i.p., s.c., oral or rectal) evaluated using a YAP camera [19] and expressed as percentage of the injected dose (% ID).

cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p21^{waf1}$, and the block of cell growth in the G_0/G_1 phase of the cell cycle [6].

In vivo antitumor activity

Biodistribution

To investigate HA-But biodistribution according to the different routes of administration, pharmacokinetics studies were performed using technetium-99 m (^{99m}Tc) labeled HA-But. ^{99m}Tc is the γ-emitting radioisotope most widely used in radiodiagnosis and in pharmacological studies as a radioactive probe. After applying an efficient labeling method to directly anchor it to HA polymer with minor changes in charge, conformation and hydrophilicity, and without significant changes in physiological interactions, solutions containing [^{99m}Tc]HA-But were administered i.v., i.p. or s.c. to healthy male CBA/Lac mice and scintigraphic images were collected every 5 min for 1 h after i.v. injection, every 30 min for 2 h after i.p. administration and every 10 min for 6 h after s.c. injection, using a YAP camera, specifically designed for the imaging analysis of the *in vivo* distribution of radiolabeled compounds.

The results (Fig. 2) indicated that just a few minutes after i.v. injection there was a substantial accumulation of HA-But in the liver [45% of the injected dose (% ID)] which became more intense after 1 h and was uniformly distributed in both hepatic lobes. Scintigraphic images indicated that HA-But accumulated also in the kidneys (2.3% ID), probably in relation to excretion of the metabolites produced by the HA degradation, and the results were confirmed by the evaluation of *ex vivo* distribution of HA-But, which showed the liver as the organ of preferential accumulation in agreement with the finding obtained with the observation that circulating HA is physiologically degraded by hepatic sinusoidal endothelial cells via the CD44 receptor. In addition, imaging analysis indicated that [^{99m}Tc]HA-But accumulated also in the spleen (2.6% ID) as expected considering the role of the spleen in HA degradation. Liver uptake decreased considerably when [^{99m}T]HA-But was administered i.p. or s.c. In fact, as shown in Fig. 2, after i.p. administration there was a reduced accumulation in the liver (23.5% ID) which further decreased after s.c. administration (0.5% ID). Interestingly, scintigraphic images collected 6 h after s.c. administration showed that 36% of injected [^{99m}Tc]HA-But was still localized at the injection site. These differences in HA-But pharmacokinetics, depending on the route of administration may be exploited to appropriately target HA-But: the i.v. route could be used for treating intrahepatic lesions, whereas the s.c. route may be more useful for treating local lesions or to partially bypass hepatic drug segregation.

To obtain complete information on HA-But biodistribution, a series of experiments were performed also administering [^{99m}Tc]HA-But orally or rectally. As shown in Figure 2, oral administration resulted in a partial retention of the drug in the stomach (21% ID) and in an accumulation in the duodenum (59.7% ID), whereas the scintigraphic images collected after 1 h after rectal administration indicated that HA-But localized preferentially in the colon (48.3% ID) with a significant retention

in the rectum (14.3% ID), suggesting a possible topical use of HA-But for treating colorectal carcinomas.

Toxicities

Acute and subacute toxicity experiments indicated that HA-But, administered i.v., i.p. or s.c. and followed up for 30 days, did not cause toxicity. In particular, when administered i.v., HA-But LD_{50} (i.e. the dose which induces a lethal effect in a 50% of the animals) was 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 LD_{50} values were, respectively, higher than 0.1 and 0.2 mg/ml and no animal died during a 30-day follow-up interval. 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, indicated a complete lack of toxicity, confirmed by the observation that no animal died during the 90-day follow-up interval.

Keeping with the main sites of localization for primary and metastatic human solid tumors, three murine experimental models were used to investigate the *in vivo* pharmacological activity of HA-But: (i) s.c. inoculated mammary tumor cells (MCa), able to induce both local and lung lesions, (ii) s.c. inoculated Lewis lung carcinoma cells (LL3), able to induce both local and lung lesions, and (iii) intrasplenic inoculated LL3 or melanoma cells (B16/F10), both able to induce intrahepatic lesions. In addition, i.p. inoculated lymphoma cells (TLX5), able to induce both i.p. ascitis and brain metastases, were also used to explore the activity of HA-But on a systemic tumor model.

HA-But and localized metastatic tumor lesions

For the evaluation of the HA-But effect in treating localized tumor lesion, CBA/Lac female mice, s.c. inoculated with 1.5×10^6 MCa cells, were intratumorally treated with 0.05 mg/ml/day for 9 days starting from day 11 after the cell inoculum. Primary tumor growth was evaluated every day and lung metastases were evaluated at the sacrifice of the animals on day 27. The intratumor treatment with a dose of HA-But free of toxicity significantly reduced primary tumor size as compared to untreated controls. Moreover, intratumor injection of HA-But 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 were obtained when the effect of HA-But was investigated in the s.c. inoculated LL3 cell 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 [19]. Even though we had no direct information on the effect of HA-But on cell motility and/or the invasive potential of the metastatic cell line used in our experiments, it is conceivable to assume that HA-But should be able to compete for the binding to CD44 receptors with the endogenous components of the ECM, and to exert a detrimental effect on cell motility and therefore on invasion potential.

HA-But and intrahepatic lesions

Very interesting results were obtained in HA-But-treated mice in which intrahepatic lesions were induced after the intrasplenic inoculation of 2×10^5 LL3 or 1×10^5 B16/F10 cells, two cell lines known for their particular aggressiveness. An intrasplenic inoculum model was chosen to mimic the biological outcome of liver metastases, avoiding the use of conventional *in vivo* experimental models which imply the production of 'artificial' liver colonization via i.v. injected tumor cells. As shown in Table 1, s.c. or i.p. administration of HA-But dramatically reduced the formation of liver metastases produced by both cell lines. In particular, with regard to 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 metastases free. A greater response rate was observed in mice 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 the animals in the s.c. or i.p. control groups. In addition, histological analysis of the liver parenchyma indicated that independent of the tumor type used, HA-But did not affect liver morphology [19]. These findings were further strengthened by a parallel series of experiments in which the effect of HA-But on survival time of mice intrasplenically implanted with 1×10^5 B16/F10 cells was investigated. Prolonged treatment with low doses of Ha-But (s.c. 0.04 mg/ml/day plus i.p. 0.01 mg/ml on days 4, 11, 18, 25 and 31) significantly increased the survival time of treated mice with respect to untreated controls. Noteworthy, 80% of HA-But-treated animals were still alive 90 days after tumor implantation versus 27% in the untreated group [19]. Since the mice treated with HA-But are still alive 12 months after the end of the experiment they may be considered disease free.

HA-But and lymphoma

With regard to the activity of HA-But on the i.p. inoculated lymphoma cells (TLX5) model able to induce both i.p. ascitis and brain metastases, in a preliminary

Table 1 Effect of 7-day i.p. or s.c. treatment with HA-But on liver metastasis formation following intrasplenic implantation of 2×10^5 LL3 or 1×10^5 B16/F10 melanoma cells in CBA/Lac female mice

Route of administration	Treatment group	Percentage of metastases-free animals	
		LL3 induced	B16-F10 induced
s.c.	control	14	0
	HA-But	86[a]	100[a]
i.p.	control	12.5	0
	HA-But	87.5[a]	100[a]

[a] $p < 0.05$, with respect to control (Fisher's exact test).

experiment we observed that with respect to controls, the s.c. treatment with HA-But at 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) not paralleled by an increased survival time, probably due to the inability of HA-But to pass the blood–brain barrier and therefore to affect brain metastases.

Conclusions and future perspectives

The studies summarized in this review provide evidence that HA-But, a novel bioconjugate constituted by a backbone of HA, one of the main components of the ECM, partially esterified with BA, the smallest HDAC inhibitor, is a potent inhibitor of cell growth *in vitro* and an antiproliferative/antimetastatic agent *in vivo*. In addition, they indicate that HA is a very suitable carrier because of its high biocompatibility, and its ability to stabilize the BA molecule and to specifically target it to tumor cells without interfering with its mechanism of action. Moreover, since CD44–HA interaction is an important requirement in promoting tumor growth and metastasis spread, an additional non-negligible effect of such a compound is represented by the disruption of this interaction by the presence of exogenous HA with a marked reduction of local neoplastic growth and dissemination. This detrimental effect on tumor growth and spread could also be mediated through the inhibition of neoangiogenesis, the formation of new blood vessels from the pre-existing vascular network, which allows the tumor mass to overcome the constraints related to the lack of the oxygen and nutrients required for its growth and spread. Through a complex mechanism of action, which implies also the synthesis of some angiogenesis-related factors, the most important of which is vascular endothelial growth factor (VEGF), tumor cells activate proliferation and migration of endothelial cells [20] that respond to the angiogenic stimulus overexpressing CD44 on their plasma membrane and moving through the ECM towards the tumor mass to be vascularized. Since we demonstrated that BA is able to modulate VEGF synthesis [21], HA-But may act also as an antiangiogenic agent.

Undoubtedly, several HDAC inhibitors, including TSA, SAHA, depsipeptide and MS-275, are very promising drugs currently in phase I and II clinical trials either as monotherapy or in combination with other cytotoxic and differentiation agents, for the treatment of solid and hematologic tumors [22–24]. 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 a high affinity for CD44, and which has been shown to be overexpressed in most human cancers, including breast, colon, lung and hepatic carcinoma, could be more useful to specifically target agents to primary as well as metastatic tumors by exploiting this CD44 overexpression. The presence of CD44 receptor on the membrane of some normal epithelial and mesenchymal cells also does not constitute a problem since, in agreement with literature data [25], we have demonstrated that in normal cells, like fibroblasts, HA-But was ineffective, suggesting that only in actively proliferating cells, like tumor cells, is the drug really effective.

The promising results obtained using HA as a carrier for BA delivery suggest further interesting development for hyaluronic butyric esters in which other small molecules, acting through a different mechanism than BA and whose biological activity could be potentiated by the presence of BA are simultaneously bound to the same HA backbone, e.g. 5-aza-2-deoxycytidine (5-AZA) retinoic acid (RA) or 1α,25-dihydroxyvitamin D_3 (DHD_3).

Among the epigenetic modifications related to tumor development there is the aberrant DNA hypermethylation by DNA methyltransferase (DNMT) of CpG islands in promoter gene regions that can lead to silencing of tumor suppressor genes or genes involved in cell growth regulation [26]. This epigenetic process, characterized by a reversible transcriptional silencing without structural genetic alterations, can be reversed *in vitro* using DNMT inhibitors, such as 5-AZA. Since the recent demonstration of the existence of molecular complexes between proteins able to specifically bind methylated CpG islands (methyl CpG-binding protein) and several members of HDAC family [26], and that HDAC inhibitor activity can be potentiated by the simultaneous presence of DNMT inhibitors with an increase in cellular susceptibility to HDAC inhibitors, a suitable evolution of the hyaluronic butyric esters could be a new chemical entity in which BA and 5-AZA are simultaneously bound to the same HA backbone. Such a compound should be able to reactivate silenced genes, and to enhance the re-expression of specific genes involved in cell growth arrest, terminal differentiation and apoptosis as supported by the accumulating evidences confirming the hypothesis that the combination of HDAC and DNMT inhibition could

be very effective in inducing apoptosis, differentiation and/or cell growth arrest in human lung, breast and colon cancer cell lines [27,28].

In two other interesting new bioconjugates, HA-But could be simultaneously bound to RA or DHD_3. These molecules are both clinically used for their differentiation activity in solid and hematologic tumors, but their use is limited by serious side-effects such as hypercalcemia (DHD_3) or drug resistance (RA) [29,30]. In the case of DHD_3, it is conceivable that the concomitant presence of another differentiation inducer, such as BA, could enhance the biological activity of DHD_3 allowing the administration of lower and therefore non-toxic doses [31]. In the case of RA, experimental and clinical findings have indicated that the simultaneous presence of an HDAC inhibitor (TSA or BA) may restore the sensitivity to retinoids [32], thus suggesting the possibility to also use this combination to obtain clinical remission in RA-resistant patients and providing the rationale for the development of a retinoic/butyric hyaluronan ester.

Acknowledgments

The authors wish to thank Drs I. Scarlata, R. Rossin and S. Zorzet who contributed, respectively, to HA-But synthesis, pharmacokinetic evaluation and *in vivo* studies.

References

1 Grunstein M. Histone acetylation and chromatin structure and transcription. *Nature* 1997; **389**:349–352.
2 Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular gene is changed in response to histone hyperacetylation. *Gene Exp* 1996; **5**:245–254.
3 Marks PA, Rifkind RA, Richon, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: causes and therapies. *Nat Rev* 2001; **1**:194–202.
4 Marks PA, Richon VM, Breslow R, Rifkind RA. Histone deacetylase inhibitors as new cancer drugs. *Curr Opin Oncol* 2001; **13**:477–483.
5 Coradini D, Pellizzaro C, Miglierini G, Daidone MG, Perbellini A. Hyaluronic acid as drug delivery for sodium butyrate: improvement of the antiproliferative activity on a breast cancer cell line. *Int J Cancer* 1999; **81**:411–416.
6 Coradini D, Pellizzaro C, Abolafio G, Bosco M, Scarlata I, Stucchi L, *et al.* Hyaluronic acid butyric esters as promising antineoplastic agents in human lung carcinoma: a preclinical study. *Invest New Drugs* 2004; **22**:207–217.
7 McIntyre A, Gibson PR, Young GP. Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. *Gut* 1993; **34**:386–391.
8 Clausen MR. Butyrate and colorectal cancer in animals and in humans. *Eur J Cancer Prev* 1995; **4**:483–490.
9 Tabuchi Y, Arai Y, Kondo T, Takeguchi N, Asano S. Identification of genes responsive to sodium butyrate in colonic epithelial cells. *Biochem Biophys Res Commun* 2002; **293**:1287–1294.
10 Coradini D, Pellizzaro C, Marimpietri D, Abolafio G, Daidone MG. Sodium butyrate modulates cell cycle-related proteins in HT29 human colonic adenocarcinoma cells. *Cell Prolif* 2000; **33**:139–146.
11 Pellizzaro C, Coradini D, Abolafio G, Daidone MG. Modulation of cell cycle-related proteins but not of p53 expression by sodium butyrate in a human non-small cell lung cancer cell line. *Int J Cancer* 2001; **91**:658–664.
12 Medina V, Edmonds B, Young GP, James R, Appleton S, Zalewski PD. Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome *c*-dependent pathway. *Cancer Res* 1997; **57**:3697–3707.
13 Miller AA, Kurschel E, Osieka R, Schmidt CG. Clinical pharmacology of sodium butyrate in patients with acute leukemia. *Eur J Cancer Clin Oncol* 1987; **23**:1283–1287.
14 Pouillart P, Cerutti I, Ronco G, Villa P, Chany C. Butyric monosaccharide ester-induced cell differentiation and anti-tumor activity in mice. Importance of their prolonged biological effect for clinical application in cancer therapy. *Int J Cancer* 1991; **49**:89–95.
15 Planchon P, Pouillart P, Ronco G, Cerutti I, Villa P, Pieri F. Differential elimination of synthetic butyric triglycerides *in vivo*: a pharmacokinetic study. *J Pharm Sci* 1993; **82**:1046–1048.
16 Aviram A, Zimrah Y, Shaklai M, Nudelman A, Rephaeli A. Comparison between the effect of butyric acid and its pro-drug pivaloyloxy-methylbutyrate on histones hyperacetylation in an HL-60 leukemic cell line. *Int J Cancer* 1994; **56**:906–909.
17 Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, *et al.* Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med* 1993; **177**:443–455.
18 Günthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, *et al.* A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991; **65**:13–24.
19 Coradini D, Zorzet S, Rossin R, Scarlata I, Pellizzaro C, Turrin C, *et al.* Inhibition of hepatocellular carcinomas *in vitro* and hepatic metastases *in vivo* in mice by the histone deacetylase inhibitor HA-But. *Clin Cancer Res* 2004; **10**:4822–4830.
20 Simeister G, Martiny-Baron G, Marmé D. The pivotal role of VEGF in tumor angiogenesis: molecular facts and therapeutic opportunities. *Cancer Metast Rev* 1998; **17**:241–248.
21 Pellizzaro C, Coradini D, Daidone MG. Modulation of angiogenesis-related proteins synthesis by sodium butyrate in colon cancer cell line HT29. *Carcinogenesis* 2002; **23**:735–740.
22 Kelly WK, Richon VM, O'Connor O, Curley T, MacGregor-Curtelli B, Tong W, *et al.* Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid (SAHA) administered intravenously. *Clin Cancer Res* 2003; **9**:3578–3588.
23 Sandor V, Bakke S, Robey RW, Kang MH, Blagoslonny MV, Bender J, *et al.* Phase I trial of the histone deacetylase inhibitor depsipeptide (FR901228, NSC630176) in patients with refractory neoplasms. *Clin Cancer Res* 2002; **8**:718–728.
24 Gojo I, Karp JE, Mann D. Phase I study of histone deacetylase inhibitor (HDI) MS-275 in adults with refractory or relapsed haematologic malignancies. *Proc Am Soc Hematol* 2002; abstr 2198.
25 Byrd JC, Shinn C, Ravi R, Willis CR, Waselenko JK, Flinn IW, *et al.* Depsipeptide (FR901228): a novel therapeutic agent with selective, *in vitro* activity against human B-cell chronic lymphocytic leukemia cells. *Blood* 1999; **94**:1401–1408.
26 Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. *Science* 2001; **293**:1068–1070.
27 Zhu W-G, Lakshmanan RR, Beal MD, Otterson GA. DNA methyltransferase inhibition enhances apoptosis induced by histone deacetylase inhibitors. *Cancer Res* 2001; **61**:1327–1333.
28 Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999; **21**:597–601.
29 Banerjee P, Chatterjee M. Antiproliferative role of vitamine D and its analogs—a brief overview. *Mol Cell Biochem* 2003; **253**:247–254.
30 Degos L, Dombret H, Chomienne C, Daniel MT, Miclea JM, Chastang C, *et al.* All-*trans*-retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia. *Blood* 1995; **85**:2643–2653.
31 Gaschott T, Stein J. Short-chain fatty acids and colon cancer cells: the vitamin D receptor–butyrate connection. *Rec Results Cancer Res* 2003; **164**:247–257.
32 Warrell Jr RP, HE LZ, Richon V, Calleja E, Pandolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J Natl Cancer Inst* 1998; **90**:1621–1625.