Inhibition of Hepatocellular Carcinomas in vitro and Hepatic Metastases in vivo in Mice by the Histone Deacetylase Inhibitor HA-But

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ABSTRACT

Purpose: The purpose is to evaluate the CD44-mediated cellular targeting of HA-But, a hyaluronic acid esterified with butyric acid (But) residues, to hepatocellular carcinoma cell lines in vitro and to hepatic tumor metastases in vivo.

Experimental Design: In vitro, the CD44-dependent cytotoxicity in two human hepatocellular carcinoma cell lines (HepB3 and HepG2) with high and low CD44 expression was investigated; in vivo, the effect on liver metastases originating from intrasplenic implants of Lewis lung carcinoma (LL3) or B16-F10 melanoma in mice was compared with the pharmacokinetics of organ and tissue distribution using different routes of administration.

Results: HepB3 and HepG2 cell lines showed different expression of CD44 (78 and 18%, respectively), which resulted in 87 and 100% metastases-free animals, respectively. HA-But tends to concentrate in the liver and spleen and appears to be a promising new drug for the treatment of intrahepatic tumor lesions.

INTRODUCTION

Liver cancer, in particular hepatocellular carcinoma, is one of the most common fatal cancers in the world (1). In addition to harboring primary tumors, the liver is often the target organ for metastatic lesions from colon (2), breast (3), or lung cancers (4). Unfortunately, treatment failure is frequent and results in high recurrence rates with short survival times (5–7). Histone deacetylase inhibitors (HDACi), including sodium butyrate, are potent inducers of cell growth arrest, differentiation, or apoptosis in a variety of transformed cell lines, including hepatocellular carcinoma, and in intrahepatic metastasis-bearing animals (8, 9). Their mechanism of action is based on their ability to inhibit the activity of histone deacetylase enzymes (HDACs); these enzymes, as opposed to histone acetyltransferases, are involved in the regulation of gene expression by determining the degree of acetylation of histones, the core proteins of the nucleosomal structure. HDAC inhibition results in reactivation of the expression of several genes, including those responsible for cell growth control and differentiation. On this basis, HDACi represent a class of potentially effective anticancer agents (10). Because a major challenge in cancer therapy is to selectively target cytotoxic agents to tumor cells and at the same time reduce the side effects of these agents, we developed HA-But, a new chemical entity constituted by a backbone of hyaluronic acid (HA) partially esterified with butyric acid (But), the smallest HDACi (11). One of the main characteristics of HA-But is its strong affinity for CD44 (12), the specific membrane receptor for metastatic lesions from colon (2), breast (3), or lung cancers (4). Unfortunately, treatment failure is frequent and results in high recurrence rates with short survival times (5–7). Histone deacetylase inhibitors (HDACi), including sodium butyrate, are potent inducers of cell growth arrest, differentiation, or apoptosis in a variety of transformed cell lines, including hepatocellular carcinoma, and in intrahepatic metastasis-bearing animals (8, 9). Their mechanism of action is based on their ability to inhibit the activity of histone deacetylase enzymes (HDACs); these enzymes, as opposed to histone acetyltransferases, are involved in the regulation of gene expression by determining the degree of acetylation of histones, the core proteins of the nucleosomal structure. HDAC inhibition results in reactivation of the expression of several genes, including those responsible for cell growth control and differentiation. On this basis, HDACi represent a class of potentially effective anticancer agents (10). Because a major challenge in cancer therapy is to selectively target cytotoxic agents to tumor cells and at the same time reduce the side effects of these agents, we developed HA-But, a new chemical entity constituted by a backbone of hyaluronic acid (HA) partially esterified with butyric acid (But), the smallest HDACi (11). One of the main characteristics of HA-But is its strong affinity for CD44 (12), the specific membrane receptor for HA, which is overexpressed in most human cancers, including liver carcinoma (13).

In the present study, we investigated the in vitro effect of HA-But on the growth of two human hepatocellular carcinoma cell lines, HepB3 and HepG2. Because the presence of CD44 receptors is a fundamental requirement for the effectiveness of HA-But, we also verified the CD44 status of the two cell lines by conventional flow cytometric analysis and using technetium-99m (99mTc)-labeled HA-But for functional analysis. 99mTc,
chosen for its optimal nuclear properties (14), was directly anchored to the HA polymer (15), thus obtaining stable labeled species ($^{99m}$Tc-HA and $^{99m}$Tc-HA-But) suitable for in vitro receptor binding experiments and for pharmacokinetic studies aimed at investigating the in vivo distribution of HA-But. Furthermore, we investigated in vivo the ability of HA-But to inhibit the development of liver metastases in mice bearing intrasplenic implants of Lewis lung carcinoma (LL3; Ref. 16) or B16-F10 melanoma (17) and to improve their overall survival.

MATERIALS AND METHODS

Chemistry. HA-But, characterized by a 0.06 degree of substitution (the ratio between the number of substituted hydroxyl groups and the number of the repeating disaccharide units of polysaccharide), was prepared as described elsewhere (11). Briefly, the esterification reaction was accomplished by means of an activated reactive form of But, i.e., an adduct between butyric anhydride and 4-dimethylaminopyridine. Before any biological evaluation, the compound was purified by preparative thin layer liquid chromatography, its structure was determined by $^1$H NMR spectroscopy, and the degree of substitution of the ester measured by capillary electrophoresis in terms of free But residues delivered from the ester by basic hydrolysis. The samples used were estimated to be at least 95% pure.

Cell Lines and Cell Growth Inhibition. The human hepatocellular carcinoma cell lines HepG2 and HepB3 were purchased from the American Tissue Culture Collection (Manassas, VA) and were maintained in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (v/v), 1% glutamine (v/v), 1% sodium pyruvate (v/v), and 1% of nonessential amino acids (v/v). The cells were passaged weekly before any biological evaluation, the compound was purified by preparative thin layer liquid chromatography, its structure was determined by $^1$H NMR spectroscopy, and the degree of substitution of the ester measured by capillary electrophoresis in terms of free But residues delivered from the ester by basic hydrolysis. The samples used were estimated to be at least 95% pure.

Mice. C57BL/6 and C57BL/6xDBA/2F1 (BD2F1) and CBA/Lac mice (Harlan & Nossan; San Giovanni al Natisone, Udine, Italy), 3–6 months old and weighing 18–20 g, were used in the experiments. The mice were bred and kept in the local animal facility. Animal experimentation was carried out according to the guidelines currently in force in Italy (DDL 116) and in compliance with the Guide for the Care and Use of Laboratory Animals.

In vivo Tumor Models. The Lewis lung carcinoma (LL3) line was originally obtained from the Tumor Depository Bank, National Cancer Institute, NIH (Bethesda, MD), and was maintained by serial biweekly passages according to the National Cancer Institute protocols. Briefly, 1 $\times$ 10$^6$ viable tumor cells of a single suspension, prepared by mincing with scissors the primary tumors from donors similarly implanted 2 weeks previously, were injected i.m. into the left calf of female C57BL/6 mice. The minced tissue was filtered through a double layer of sterile gauze, centrifuged at 200 $\times$ g for 10 min, and resuspended in an equal volume of sterile PBS; viable cells were counted by the Trypan Blue exclusion test.

B16-F10 melanoma cells, originally obtained from the Tumor Depository Bank, National Cancer Institute, NIH, were cultured in modified Eagle’s medium supplemented with 10% FBS, 1% nonessential amino acids, 1% sodium pyruvate, 1% l-glutamine (100x), 1% standard antibiotics (all from Euroclone), 2% NaHCO$_3$, and 1% glucose. Cells were maintained in a humidified 5% CO$_2$ atmosphere at 37°C. The human hepatocellular carcinoma cell lines HepG2 and HepB3 were purchased from the American Tissue Culture Collection (Manassas, VA) and were maintained in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (v/v), 1% glutamine (v/v), 1% sodium pyruvate (v/v), and 1% of nonessential amino acids (v/v). The cells were passaged weekly before any biological evaluation, the compound was purified by preparative thin layer liquid chromatography, its structure was determined by $^1$H NMR spectroscopy, and the degree of substitution of the ester measured by capillary electrophoresis in terms of free But residues delivered from the ester by basic hydrolysis. The samples used were estimated to be at least 95% pure.

Flow Cytometric Analysis. The expression of CD44 receptors was investigated by flow cytometry using a murine monoclonal antibody raised against human CD44 (clone 5F12; Neo Markers, Labvision, Fremont, CA) for HepG2 and HepB3 human cell lines or a rat antimouse CD44-FITC monoclonal antibody (2 p.g; SouthernBiotech Associates, Inc., Birmingham, AL) for B16-F10 and 3LL murine cell lines. Cells were recovered from the flasks with trypsin-EDTA solution, washed with PBS, and centrifuged at 800 $\times$ g for 5 min. Parallel samples (1 $\times$ 10$^6$ cells each) were loaded, and cells were incubated with primary antibody at a dilution of 1:20 for 60 min at room temperature. The cells were then washed with PBS, centrifuged, and incubated with a secondary FITC-conjugated goat antimouse antibody (Sigma, St. Louis, MO) 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. After immunofluorescence staining, the cells were centrifuged and resuspended in a solution containing propidium iodide (5 g/ml), RNase (10 kU/ml; Sigma), and NP40 (0.005%). The fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser at 488 nm wavelength excitation and a 610-nm filter for propidium iodide fluorescence detection. The fluorescence signal was collected in linear and logarithmic mode. Data were acquired and processed using the LYSIS II software (Becton Dickinson); at least 30,000 events were recorded for each sample.

Direct Labeling of HA and HA-But with $^{99m}$Tc. HA and HA-But were labeled with $^{99m}$Tc, the most widely used $\gamma$-emitting radioisotope for radiodiagnostic purposes. Because of its optimal nuclear properties (E = 140 keV, T$_{1/2}$ = 6.02 h), which allow efficient detection by means of gamma cameras (18), $^{99m}$Tc has recently been used as a radioactive probe also in pharmacological studies to investigate the in vivo distribution of...
new drugs (19, 20). When the target molecule is a high molecular weight homopolymer, $^{99m}$Tc labeling leads to minor changes in charge, conformation, and hydrophilicity, without significant changes in biodistribution and physiological interactions. Because HA and HA-But contain many functional groups, useful to bind the $^{99m}$Tc(V)oxo core (carboxylic, hydroxyl, and amino groups), a direct method to label the two polymeric species with $^{99m}$Tc was applied (14, 21). Briefly, $^{99m}$Tc-pertechnetate, freshly eluted from a $^{99}$Mo/ $^{99m}$Tc generator (Nycomed-Amersham-Sorin, Saluggia, Italy), was reduced with 1 $\mu$mol of a 0.1 M SnCl$_2$ solution (in 0.1 M HCl) in the presence of sodium gluconate (1 $\mu$mol of a 10$^{-2}$ M solution) as exchange ligand. The polymeric species (3 mg) was then added, the pH was adjusted to 4.0 with 10$^{-2}$ M HCl, and the labeling mixture was gently mixed at 50°C for 1.5 h. All radioactivity measurements were carried out using a Dose Calibrator (M2316 Messel- tronik; Dresden). Labeling yield determination and $^{99m}$Tc-pol- tigraphic images were elaborated by a dedicated software con- nected to the hardware as described elsewhere (18). In vivo distribution of $^{99m}$Tc-HA-But was evaluated in healthy male CBA/Lac mice after i.v., s.c., or i.p. administration. Briefly, mice previously anesthetized with ketamine hydrochloride (125 mg/kg, i.p.) were injected i.v., s.c. (at two abdominal sites), or i.p. with 200 $\mu$L, 2 $\times$ 250 $\mu$L or 250 $\mu$L of a HA-But saline solution containing $^{99m}$Tc-HA-But (13, 14), and 12 MBq, respectively). Scintigraphic images of the mice’s abdomens were collected by the YAP camera for 1 h after i.v. injection, for 6 h after s.c. administration, and for 3 h after i.p. injection. At the end of image collection, the animals were sacrificed, the ex vivo distribution was evaluated, and organ radioactive accumulation was measured.

**Intrasplicen Implantation and Liver Metastasis Counting.** Intrasplicen injection of tumor cells was performed in a UV sterile fume hood. Thirty-two female BD2F1 mice were anes- thetized with an i.p. injection of 70 mg/kg Zoletil (Virbac, Milan, Italy), and their spleens were exposed through a small left flank incision, then $2 \times 10^5$ viable LL3 carcinoma cells/50 $\mu$L Matrigel (150 $\mu$g/ml; Becton Dickinson) or $1 \times 10^5$ B16-F10 melanoma cells/50 $\mu$L Matrigel were implanted. Liver metastases were allowed to grow for 4 days, then groups of eight animals were treated i.p. or s.c. with 0.6 $\mu$mol (250 $\mu$L/mouse) HA-But, a dose free of toxicity in mice as demonstrated by separate experiments with both acute and chronic treatment (data not shown); the mice were treated daily until day 10. Control animals were treated i.p. or s.c. with an equal volume of 0.9% NaCl. On day 15 (LL3) or day 21 (B16-F10) after intrasplicen cell implantation, control and treated animals were sac- rificed, and the formation of liver metastases was assessed as the number of neoplastic lesions counted by a low-power stereo microscope.

**Histological Evaluation.** To evaluate the incidental ef- fect of HA-But on the morphology of the live parenchyma, livers of control and treated groups were removed, fixed in 10% formalin, and embedded in paraffin. Four-μm sections were stained by Cajal-Gallego procedure, a triple staining method that facilitated the identification of hepatocyte nucleus/cytoplasm and extracellular matrix, and the liver morphology was micro- scopically evaluated.

**Survival Evaluation.** The effects of HA-But on the life expectancy of tumor-bearing animals was investigated in a parallel series of experiments in which female BD2F1 mice that had been intrasplicen implanted with $1 \times 10^5$ B16-F10 melanoma cells were s.c. injected with 0.15 $\mu$mol (125 $\mu$L/mouse/day) HA-But starting on day 4 and continuing until day 32 (the time at which the first control animal died), with an additional i.p. administration (12 $\mu$mol/mouse) on days 4, 11, 18, 25, and 31. The survival of the animals was recorded up to the death of the last animal.

**Statistical Analysis.** Experimental data were subjected to computer-assisted statistical analysis, and the statistical sig-
nificance of differences between IC<sub>50</sub> values in the different cell lines was assessed by the two-tailed Student’s t test, whereas the effect of HA-But on the formation of metastases was evaluated by Fisher’s exact test. Overall survival was evaluated by Kaplan-Meier product limit survival analysis and the log rank test. Two-sided P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cell Surface Expression of CD44, Cellular Uptake of 99mTc-HA-But, and Cell Growth Inhibition. Flow cytometric analysis showed that only 18% of HepG2 cells expressed CD44 receptors on their surface, whereas 78% of HepB3 cells were CD44 positive (Fig. 1A). As shown in Fig. 1B, time course experiments using the radiolabeled compound indicated that the two cell lines had different binding capability: the HepB3 binding activity increased in a time-dependent manner and reached a plateau ~6 h after treatment, whereas HepG2 had limited binding activity that started to decrease already after 4 h. Notwithstanding the presence of a different CD44 expression, HA-But was able to inhibit cell proliferation in both cell lines. In fact, as shown in Fig. 2 (top panels, as a function of mM butyric concentration; bottom panels, as a function of mg/ml concentration), after 6 days of treatment, it had a dose-dependent inhibitory effect: in HepB3 cells, at the highest concentration evaluated (4 mg/ml corresponding to 1 mM butyric concentration), HA-But induced almost complete inhibition of cell growth (90%), whereas in the CD44-poor HepG2 cells, moderate inhibition (60%) was observed, suggesting that after a sufficiently prolonged treatment interval (in this case 6 days), HA-But was effective also in CD44-poor tumor cells. A possible explanation could be the very rapid turnover of the CD44 receptor after HA-But uptake, which guarantees a continuous inhibitory intracellular concentration of the drug as demonstrated in a previous study (12). However, even if CD44 is the major receptor for HA, other membrane receptors, including the receptor for hyaluronate-mediated mobility, could be responsible for HA-But internalization. Interestingly, HA-But was more effective than NaB alone, with a 10-fold decrease in the IC<sub>50</sub> value (2.1 versus 0.12 mM and 3 versus 0.31 mM, P < 0.01, in HepG2 and HepB3 cells, respectively), which would suggest that the use of HA as a carrier for sodium butyrate can significantly improve its biological activity without any apparent negative effects due to the large dimension of the molecule.

In vivo Distribution of 99mTc HA-But. An efficient 99mTc-labeling method (85–95% labeling yield) allowed us to obtain a very stable 99mTc-HA-But complex in which the partial functionalization of hydroxyl groups with butyl ester residues did not significantly influence the labeling yield. In vivo analysis of the HA-But distribution was performed by administering i.v., s.c., or i.p. a solution containing 99mTc-HA-But to healthy anesthetized male CBA/Lac mice. With regard to i.v. injection, the scintigraphic images, at any time and with a 10-min acquisition by the YAP camera (Fig. 3B), showed a very rapid and relevant liver uptake of radiolabeled HA-But uniformly distributed in both lobes. Uptake was visible after a few minutes and more intense after 1 h (Fig. 3B); partial accumulation of the compound was seen in the kidneys, probably related to excretion of the fragments produced by polymer degradation. The result was confirmed by the postimaging distribution evaluation, which showed the liver to be the organ of preferential accumulation (46.52% of the injected dose). This is in agreement with

Fig. 1 Cell surface expression of CD44 and cellular uptake of 99mTc-HA-But. The cell surface expression of CD44 of both hepatocellular carcinoma cell lines (A) was evaluated by flow cytometric analysis. HepG2 and HepB3 cells (1 × 10<sup>6</sup>) were first incubated with a murine anti-human monoclonal antibody raised against human CD44 and then with goat FITC-labeled antimouse secondary antibody. Negative control (■) and stained samples (▲) are shown. For time course, cellular 99mTc-HA-But uptake evaluation HepG2 and HepB3 cells were seeded in chamber slides (5 × 10<sup>3</sup> cells/chamber) and grown until the exponential phase (B). Purified 99mTc-HA-But, diluted with native cold polymer to obtain a 5 mg/ml concentration, was then added and cells were incubated for 6 h at 37°C.
the findings reported by Gustafson et al. (22), who observed a similar distribution for the native HA. It is also in agreement with the observation that circulating HA is physiologically degraded by hepatic sinusoidal endothelial cells via the CD44 receptor (23). Postimaging analysis indicated noticeable 99mTc-HA-But accumulation also in the spleen, as expected given the role of the spleen in HA degradation (24). In fact, considering the amount of 99mTc-HA-But accumulated/g of tissue (expressed as percentage of the injected dose/g tissue, %ID/g) and thus taking into account the size of each organ, the spleen accumulated 11.66%ID/g, an amount consistent with that found in the liver, which accumulated 15.83%ID/g.

The high liver uptake observed after i.v. injection decreased considerably when 99mTc-HA-But was given i.p. or s.c. Intraperitoneal administration reduced 99mTc HA-But accumulation in the liver to 12.33%ID/g, which additionally decreased to 0.47%ID/g after s.c. administration. Interestingly, after i.p. injection splenic accumulation of 99mTc HA-But was similar to that following i.v. administration (11.08%ID/g), whereas the liver uptake was reduced to 5.21%ID/g. This would imply that additional studies are necessary to evaluate the possible activity of HA-But in hyperproliferative diseases of the spleen. Lastly, scintigraphic images collected 6 h after s.c. administration showed that 36%ID/g 99mTc HA-But was still localized at the site of injection (Fig. 3C). Differences in HA-But pharmacokinetics depending on the route of administration could be exploited to appropriately target the drug: the i.v. route could be used for treating intrahepatic lesions, whereas the s.c. route might be more useful for treating local or splenic lesions or even to partially bypass the hepatic drug segregation. Mahter et al. (25) demonstrated that the normal/tumor ratio HA distribution decreased by pretreating the animals with chondroitin sulfate due to the saturation of the normal hepatic sinusoidal cells. This observation supports our finding in a similar study (data not shown) where we demonstrated a different organ distribution of HA-But when drug administration was preceded by presaturation with chondroitin sulfate and suggests that it may be possible to treat not only hepatic lesions but also tumors in other organs.

**HA-But Inhibits Formation of Liver Metastases.** Most of the available in vivo experimental models of liver metastases imply the production of artificial liver colonization via i.v.-injected tumor cells. To use an in vivo model more similar to the biological outcome of liver metastases, we chose to intrasplenically implant experimental tumor cells. We used LL3 carcinoma and B16-F10 melanoma cells, two murine tumors that had previously been shown to produce liver metastases after intrasplenic injection (26, 27) and that expressed a high percentage of CD44-positive cells (68 and 87%, respectively; Fig. 4), in agreement with the recent finding by Mummert et al. (28). The effect of 7-day i.p. or s.c. administration of 0.6 mmol of HA-But (a nontoxic dose to the host, as demonstrated in separate studies) on the formation of liver metastases is reported in Table 1. HA-But reduced liver colonization, with a statistically significant difference compared with the control group (P < 0.05). In
Fig. 3 In vivo distribution of \(^{99m}\text{Tc} \) HA-But was investigated using a yttrium-aluminum-perovskite camera (A), a high-spatial resolution gamma camera. B and C show the scintigraphic images of mouse abdomens obtained 1 h after i.v. (B) or 6 h after s.c. (C) administration of a HA-But saline solution containing \(^{99m}\text{Tc} \) HA-But. Arrows indicate the main sites of accumulation: 46% of the injected dose in the liver after i.v. administration and 36% of the injected dose at the site of injection after s.c. administration.

Fig. 4 Cell surface expression of CD44, evaluated by flow cytometry, of B16-F10 and 3LL cells used in in vivo experimental model.
particular, when LL3 cells were considered, six of seven (86%) of the s.c.-treated animals and seven of eight (87.5%) of the i.p.-treated animals were free of macroscopically detectable metastases, and only one animal/treatment group (i.p. or s.c.) presented metastatic foci at sacrifice (i.e., 15 days after implantation). Conversely, in the untreated control group only one of seven (14%) of the s.c.-treated animals and one of eight (12.5%) of the i.p.-treated animals were metastases free (Table 1). A similar response rate was observed 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 zero of seven animals in both (s.c. and i.p.) control groups. A representative photograph of liver sections from formalin-fixed and paraffin-embedded livers obtained from each treatment group was stained with the Cajal-Gallego method and examined. No signs of liver damage due to HA-But treatment were observed.

### Table 1 Effect of 7-day i.p. or s.c. treatment with HA-But on liver metastasis formation after intrasplenic implantation of LL3 carcinoma or B16-F10 melanoma cells

<table>
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<th>Route of administration</th>
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<sup>a</sup> P < 0.05, with respect to control (Fisher’s exact test).

very effectively inhibited liver metastasis formation, although it was not administered via the optimal route (i.v.).

**Increased Life Expectancy in Animals Treated with HA-But.** For this experiment, we used the B16-F10 melanoma model because of the better results obtained in previous tests and of the very high CD44 positivity of these tumor cells. Prolonged treatment with HA-But for up to 12 days with low doses of HA-But significantly (P < 0.03) increased the life expectancy of BD2F1 mice over untreated controls (Kaplan-Meier analysis; Fig. 6). In addition, 90 days after tumor implantation, 8 of 10 HA-But treated animals were still alive versus 3 of 11 (27%) in the untreated group.

In conclusion, the present results indicate that HA-But, a compound in which HA is esterified with butyric ester and which has been previously reported to block lung cancer growth and spread (11), could be useful also for the selective treatment
of intrahepatic lesions. Undoubtedly, the use of HDACi as anticancer agents is not new and some of these agents (phenylbutyrate, suberoylanilide hydroxamic acid, MS-275, and depsipeptide) are currently included in Phase I/II clinical trials for the treatment of solid tumors, lymphomas, and multiple myeloma (Refs. 29–31; additional information online). However, the use of these drugs does not allow the achievement of the major goals in cancer therapy: to selectively target anticancer molecules to organs or compartments harboring tumor cells. Conversely, HA-But, which has strong affinity for the CD44 membrane receptor (12), could be a promising antineoplastic agent for the treatment of primary as well as metastatic intrahepatic cancers, especially taking into consideration that anti-metastatic effects have been obtained using a nonoptimal route of administration for the liver (as demonstrated by the limited accumulation observed in distribution analysis); this could mean that it might be possible to administer lower doses of the compound i.v. to obtain a therapeutic effect.

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