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(54) **Title:** THE HISTONE DEACETYLASE INHIBITOR HELMINTHOSPORIUM CARBONUM TOXIN FOR SUPPRESSING MALIGNANT QUALITIES OF NEUROBLASTOMA CELLS

(57) **Abstract:** The present invention relates to treating a subject who has a small round blue cell tumor with a histone deacetylase inhibitor, particularly with an inhibitor that is Helminthosporium Carbonum toxin. Helminthosporium carbonum toxin reverses in nano mo lar dosage tumor cells from malignant towards benign. Conversion to benign is associated with activation of retinoblastoma tumor suppression networks. Stimulating tumor suppression pathways presents a novel approach to small round blue cell tumor therapy.

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THE HISTONE DEACETYLASE INHIBITOR HELMINTHOSPORIUM CARBONUM TOXIN FOR SUPPRESSING MALIGNANT QUALITIES OF NEUROBLASTOMA CELLS

FIELD OF THE INVENTION

The present invention relates to treating a subject who has a small round blue cell tumor with a histone deacetylase inhibitor, particularly with an inhibitor that is *Helminthosporium carbonum* toxin. *Helminthosporium carbonum* toxin reverses in nanomolar dosage tumor cells from malignant towards benign. Conversion to benign is associated with activation of retinoblastoma tumor suppression networks. Stimulating tumor suppression pathways presents a novel approach to small round blue cell tumor therapy.

BACKGROUND

Despite state-of-the-art multimodal therapy, the dismal prognosis of high-risk neuroblastoma (NB) with an overall survival rate of only 34 % makes the development of novel therapeutic strategies indispensable. Almost all traditional cytotoxic chemotherapeutic agents target tumor cell growth by (i) causing DNA damage, (ii) interfering with the mitotic spindle organization or (iii) modulating enzymatic activity involved in ribonucleotide synthesis and DNA replication. The underlying principle of all such approaches inadvertently affects not only cancer cells but also untransformed dividing cells, which results in increased toxicity to normal tissue. In the case of NB, it considerably contributes to long-term complications in 44 % of the survivors of high-risk NB . Not bypassing neoplastic mechanisms but activating tumor suppression pathways presents a novel approach to NB therapy.

The transcriptional machinery regulating cell quiescence, as well as its transition through the cell cycle, depends heavily on the growth inhibitory function of the tumor suppressor RB, which controls a number of downstream effector cascades including the E2F transcription factor family and the RB-Skp2-$p27^{kip1}$ pathway. In neuronal tissues, RB not only controls mitotic processes but also governs cell fate specification and differentiation during neurogenesis (37-41). This notion is supported by the ability of RB to bind a broad spectrum of varying proteins

including tissue-specific transcription factors (42). RB is known to play some role in neuronal development orchestrating and coupling the complex processes of cell division and maturation (43).

It also is known that RB function is impaired in numerous human cancers of all age classes by different oncogenic events (21). In neuroblastoma, a clear correlation was identified between poor patient prognosis and deregulated E2F effector cascades (22) as well as an impaired RB-Skp2-p27^{kip1} pathway (Westermann et *al.*, unpublished data). Neuroblastoma originates from progenitors of mature sympathoadrenal cells and is characterized by marked cellular heterogeneity (2).This is reflected by considerably diverse clinical behaviors with at least three different courses including spontaneous regression of clinically apparent NB, differentiation into mature ganglioneuroma and, finally, aggressive NB characterized by progression despite state-of-the-art multimodal therapy. An overall survival rate of only 34 % in stage IV NB emphasizes the need for novel more biologically based therapeutic strategies activating pathways known to mediate tumor suppression.

Specific genomic DNA aberrations such as *MYCN* oncogene amplification (4) as well as deletions or gains of distinct chromosomal regions including 1p (5), 11q (6) and 17q (7) have been identified and may be involved in NB genesis. However, the epigenetic mechanisms navigating the orchestrated expression of the many signals involved in migration and terminal differentiation during neural development are poorly understood. Aberrant DNA methylation and changes in the acetylation and methylation pattern of lysine residues in the tails of nucleosomal core histones alter chromatin structure and gene expression (8). Recently, an overall loss of monoacetylation at H4-Lys16 and trimethylation at H4-Lys20 located at hypomethylated DNA repetitive loci was identified in both primary tumors and a panel of human cancer cell lines including the NB cell line SKN-SH (9). Thus, epigenetic deregulation during differentiation of the sympathoadrenal lineage may disturb neural differentiation and lead to NB genesis.

Changes in the epigenetic pattern of tumor cells triggering profound deregulation of gene expression are susceptible to remodeling by specific drug targeting. Small-molecule histone deacetylase inhibitors (HDACIs) with diverse chemical structures and pharmacologic properties interfere with HDAC-activity in transformed cells *in vitro* (10) initiating anti-proliferative (11), pro-apoptotic (12) and pro-differentiating (13) effects.

It is well known that recruitment of HDACs to promoters of E2F target genes is initiated by RB to attenuate cell proliferation. Previously, it has been speculated that HDACIs may undesirably antagonize RB mediated cell cycle control by interfering with the repression of S-phase genes through HDAC recruitment (48). Indeed, it has been shown that treatment of SAOS2 osteosarcoma cells with the HDACI trichostatin A substantially reduces the growth inhibitory effects of RB (46). Recently, it has been demonstrated that RB recruits HDACs to specific promotors including those of cyclin A, Cdc2, topoisomerase IIα and thymidylate synthase and subsequently represses transcriptional activity . However, cell cycle inhibitory action of RB was not intrinsically dependent upon the ability to recruit HDAC activity to promotors of target genes initiating cell cycle arrest . HDAC-independent repression mechanisms such as altering SWI/SNF chromatin remodeling activity were found to play a major role in RB mediated cell cycle arrest.

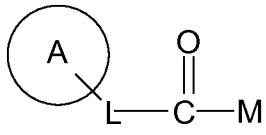
Phase I/ II clinical trials elucidating HDACI action against refractory solid tumors, leukemias and lymphomas show for several HDACIs encouraging results including relatively low toxicity . Carboxylates such as sodium phenylbutyrate and valproic acid elicit growth arrest both in *in vitro* NB models and in NB tumor bearing mice. However, poor potency and low bioavailability (17) became apparent in clinical trials making them less desirable for NB treatment. No clinical responses but unfavorable pharmacokinetic properties and cumulative bone marrow toxicity are the outcomes of a clinical trial investigating the benzamide MS-275 previously shown to possess anti-NB activity both *in vitro* and in a xenograft murine model of NB.

SUMMARY OF THE INVENTION

In one aspect of the present invention is a method of treating a subject having a tumor, comprising administering a dose of a histone deacetylase (HDAC) inhibitor to a subject, wherein the HDAC inhibitor activates tumor suppression signalling networks. The present invention also encompasses the use of a HDAC inhibitor for the preparation of a medicament for the treatment of tumors, wherein the HDAC inhibitor activates tumor suppression signalling networks. In one embodiment, the tumor suppression pathway is a retinoblastoma (RB) tumor suppression pathway.

In one embodiment, the HDAC inhibitor is selected from a group consisting of carboxylates, hydroxamic acid derivatives, benzamides and cyclic peptides, in particular tetracyclic peptides. In a preferred embodiment, the HDAC inhibitor is *Helminthosporium Carbonum* toxin.

In one embodiment, the HDAC inhibitor is a compound according to the general formula (I)



(I)

In the most general meaning classified below under A, the symbols have the following meanings:

A:

A is a 9- to 15-membered heterocycle optionally carrying one or more double bonds, and comprising 3 to 5 units of the type -D-E-G- wherein

D is selected from $-N(R^1)-$, -O-, and $-S(O)(R^2R^3)_m-$; and/or

E is selected from methylene $-CH_2-$, ethylene $-CH_2CH_2-$ and propylene $-CH_2-CH_2-CH_2-$, wherein in the methylene, ethylene and propylene groups one or more H atoms can be replaced by: linear and branched C_1- to C_{10} alkyl groups, which are optionally substituted by one or more substituents from the group -OH, F, -SH, $-SCH_3$, $-NH_2$, $-NHC(NH)NH_2$, $-C(O)OH$, $-C(O)NH_2$, C_6H_5, C_6H_4OH, indolyl, imidazolyl or a salt thereof; and linear and branched C_2- to C_{10} alkylene groups, which are optionally substituted by one or more OH and/or one or more F; or, in case 2 or more even-numbered substituents are present, the named alkyl or alkylene group forms together with a further alkyl or alkylene group on the same or a different carbon atom a 5- to 7-membered cycle; or the named alkyl and alkylene groups form together with the group R^1 a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

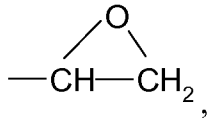
G is a keto group -C(O)- or a thioketo group -C(S)-; and/or

wherein the units -D-E-G- not attached to L can be fully or partially identical with or entirely different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

L is attached to D or E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group in which one or more carbon atoms may be replaced by non adjacent O atoms, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



hydroxyamino groups –N(OH)H, hydroxymethyl groups -CH_2OH, 1-fluoroethyl groups -$CHFCH_3$ and 1-chloroethyl groups -$CHClCH_3$; and/or

R^1 is selected from: H; linear and branched C_1- to C_{10} alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear or branched C_2- to C_{10} alkylene groups which are optionally substituted by one or more OH and/or one or more F; or R^1 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

R^2 and R^3 are independently of each other selected from: H; linear and branched C_1- to C_{10} alkyl groups which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_{10} alkylene groups, which are optionally substituted by one or more OH and/or one or more F; or R^2 and R^3, together with the sulfur atom to which they are attached, form a 5- to 7-membered cycle; or one of R^2, R^3 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

m is 0 or 1,

and/ or a pharmaceutically acceptable salt thereof.

L is always attached to E in one of the units –D-E-G-, which unit has to be different from the other units –D-E-G-.

The cycle A comprises 3, 4 or 5 amino acids. Suitable amino acids are known to the person skilled in the art and comprise natural amino acids and synthetic amino acids. Natural amino acids are those occurring in nature and comprise: glycine, alanine, serine, cysteine, phenylalanine, tyrosine, tryptophane, threonine, methionine, valine, proline, leucine, isoleucine, lysine, arginine, histidine, aspartic acid, asparagine, glutamic acid, glutamine. The named amino acids are found in the L form in nature. In the context of the present invention, the above referenced natural amino acids in the L-form and in the D-form are preferred.

The double bonds in the cycle A may be a result of enol forms resulting from tautomerism. In case enol forms result from tautomerism between the keto group and a neighbouring -NH or a -CH function, the enol forms may be stabilised or fixed by alkylation. Suitable alkylation methods are known to the person skilled in the art.

The present invention also includes prodrugs of the compounds of formula (I). Examples for suitable prodrugs include molecules according to formula (I) as defined beforehand, and wherein the epoxy function is replaced by an appropriate precursor function therof, e.g. a vicinal diol function, a chlorohydrine function -CH(OH)(Cl)CH- or an olefinic double bond.

Preferred embodiments are classified below under B:

B:

A is a 11- to 13-membered cycle; comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein one or both H atoms can be replaced by: a linear or branched C_1- to C_4 alkyl group, which are optionally substituted by one or more substituents from the group -OH, F, -SH, $-SCH_3$, $-NH_2$, $-NHC(NH)NH_2$, $-C(O)OH$, $-C(O)NH_2$, C_6H_5, C_6H_4OH, indolyl, imidazolyl or a salt thereof; and/or a linear or branched C_2- to C_4 alkylene group, which is optionally substituted by one or more OH and/or one or more F; or the named alkyl and alkylene groups form together with the group R^1 a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

G is a keto group -C(O)-; and/or

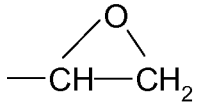
wherein the units -D-E-G- not attached to L can be fully or partially identical with or entirely different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is selected from: H; linear and branched C_1- to C_4 alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_4 alkylene groups which are optionally substituted by one or more OH and/or one or more F; or R^1 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached, and/or

L is attached to E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group in which one or more carbon atoms may be replaced by non adjacent O atoms, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



and hydroxyamino groups –N(OH)H.

The cycle A comprises 4 natural amino acids in the L- or D-form, selected from those cited beforehand.

Even more preferred embodiments are classified below under C:

C:

A is a 12-membered cycle and comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein in one or two units -D-E-G-, one or both H atoms can be replaced by: a linear or branched C_1- to C_4 alkyl group and/or a linear or branched

C_2- to C_4 alkylene group; and wherein in only one unit -D-E-G-, one H atom in -CH_2- is replaced by a linear or branched C_1- to C_4 alkyl group or a linear or branched C_2- to C_4 alkylene group, with the said alkyl or alkylene group forming together with R^1 in the neighbouring NR^1 group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

G is a keto group -C(O)-; and/or

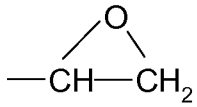
at least 2 units -D-E-G- are different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is selected from: H; linear and branched C_1- to C_4 alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_4 alkylene groups, which are optionally substituted by one or more OH and/or one or more F; and wherein in exactly one unit -D-E-G-, R^1 forms together with the said alkyl or alkylene in the neighbouring –CH_2- group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

L is attached to E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



and hydroxyamino groups –N(OH)H; and/or

the cycle A comprises 4 natural amino acids in the L- or D-form.

Embodiments, which are still preferred over those under C, are classified below under D:

D:

A is a 12-membered cycle and comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein in one or two units -D-E-G-, one or both H atoms can be replaced by: a methyl or ethyl group; and wherein in only one unit -D-E-G-, one H atom in $-CH_2-$ is replaced by a linear or branched C_1- to C_4 alkyl group or a linear or branched C_2- to C_4 alkylene group, and wherein the said alkyl or alkylene group forms together with R^1 in the neighbouring NR^1 group a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached;

G is a keto group -C(O)-; and/or

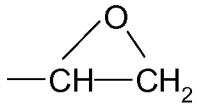
wherein at least 2 units -D-E-G- are different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is H or a linear or branched C_1- to C_4-alkyl group in three of the units -D-E-G-; and wherein in the other unit –D-E-G, R^1 forms together with the said alkyl or alkylene in the neighbouring $-CH_2-$ group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

L is attached to E in one unit -D-E-G- and is a C_3- to C_5-alkyl group, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups

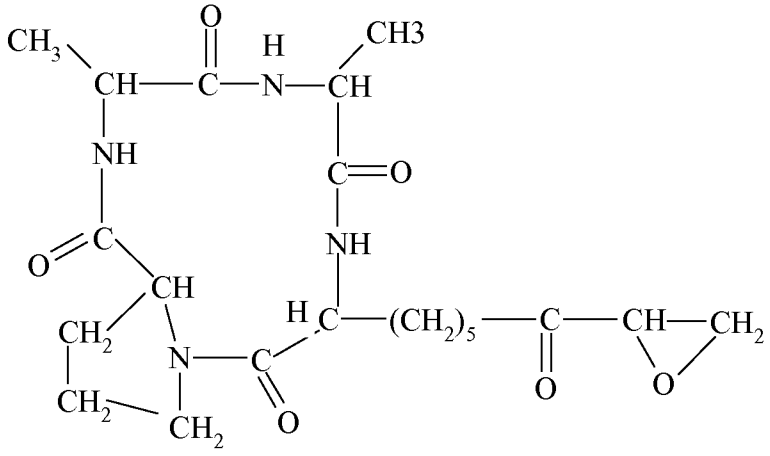


and hydroxyamino groups –N(OH)H; and/or

the cycle A comprises 4 natural amino acids in the L- or D-form.

Within the definitions made beforehand for embodiments A, B, C and D, the individual meanings for each substituent or each group are not construed to be restricted to the embodiment under which it is mentioned, i.e. a definition cited under C my be combined with the definitions made under e.g. A, B, or D. The entire combination of all the definitions for each substituent made under each of A, B, C and D is however preferred.

As an example, for the compounds according to the general formula (I), reference is made to formula (II), i.e. *Helminthosporium carbonum* toxin (HC-Toxin).



(II)

The compound of the general formula (II) is known as such and does as a compound not belong to the present invention, only with respect to its particular pharmacological and biological properties, as laid out hereinafter.

In a further embodiment, the present invention relates to a compound according to formula (I) as defined above in general form or in preferred embodiments, or a pharmaceutically acceptable salt thereof, for use as medicament. In still a further embodiment, the present invention relates to a compound according to formula (I) as defined above in general form or in preferred embodiments, or a pharmaceutically acceptable salt thereof, for the manufacture of a pharmaceutical composition for the treatment of the tumors or cancer diseases recited herein and, preferably, the Small Round Blue Cell Tumor. Small Round Blue Cell Tumor is, preferably, a pediatric soft tissue tumor. A Small Round Cell Tumor as usd herein, preferably, is a tumor selected from the group consisting of: Desmoplastic small round blue cell tumor, Ewing's Sarcoma, Acute Leukemia, Small Cell Lung Carcinoma, Small Cell Mesothelioma, Neuroblastoma, Primitive Neuroectodermal tumor, Rhabdomyosarcoma, Wilm's Tumor, and Melanoma, most preferably, it is a neuroblastoma.

The term „pharmaceutical composition" is sometimes referred to as „pharmaceutical" or „medicament" hereinafter or in the prior art. Said terms shall have the same meaning and may be used interchangeably.

In one embodiment, the tumor suppression pathway is the retinoblastoma tumor suppression pathway. In another embodiment, the cancer is a Small Round Blue Cell Tumor. In a further embodiment, the Small Round Blue Cell Tumor is selected from the group consisting of Desmoplastic small round blue cell tumor, Ewing's Sarcoma, Acute Leukemia, Small Cell Lung Carcinoma, Small Cell Mesothelioma, Neuroblastoma, Primitive Neuroectodermal tumor, Rhabdomyosarcoma, Wilm's Tumor, and Melanoma. In a preferred embodiment, the Small Round Blue Cell Tumor is a Neuroblastoma.

In one embodiment, the subject is a mammal. In a further embodiment, the mammal is a human, primate, rat, dog, cat, cattle, mouse, guinea pig, gerbil, pig, or sheep. In a preferred embodiment, the subject is a human.

In one embodiment, the dose of the HDAC inhibitor administered to the subject is about 1 nM, about 2 nM, about 3 nM, about 4 nM, about 5 nM, about 6 nM, about 7 nM, about 8 nM, about 9 nM, about 10 nM, about 11 nM, about 12 nM, about 13 nM, about 14 nM, about 15 nM, about 16 nM, about 17 nM, about 18 nM, about 19 nM, about 20 nM, about 21 nM, about 22 nM, about 23 nM, about 24 nM, about 25 nM, about 26 nM, about 27 nM, about 28 nM, about 29 nM, about 30 nM, or more than about 30 nM, or any integer there in between. In one embodiment, the HDAC inhibitor which is administered at this dose is a peptide according to formula (I), preferably a tetrapeptide, in particular *Helminthosporium Carbonum* toxin.
In one embodiment, the dose of the HDAC inhibitor administered to the subject is between 1-5 nM, between 5-10 nM, between 10-15 nM, between 15-20 nM, between 20-25 nM, between 25-30 nM or more than 30 nM, e.g., between 30-40 nM or 40-50 nM. In a preferred embodiment, the dose is between 10-20 nM.
In a preferred embodiment, the HDAC inhibitor which is administered to the subject is *Helminthosporium Carbonum* toxin at a dose concentration of between 10-20 nM.

Both the foregoing general description and the following brief description of the drawings and the detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D. HC-toxin efficiently mediates growth arrest and apoptosis in NB cells with *MYCN* single copy status and with transfected human *MYCN* oncogene governing enhanced expression. Figure 1A: Time-course and dose-response growth curves of SH-EP NB cells cultured with HC-toxin or MeOH solvent control. Induction of histone 4 acetylation (panAc-H4) by HC-toxin is shown in the *upper panel*. Figure 1B: induction of G_0/G_1 cell cycle arrest and apoptosis (sub-G_1) in SH-EP NB cells by HC-toxin. Shown *at right* are exemplarily FACS profiles of solvent control and HC-toxin treated cells after 72 h of exposure. Striped areas represent S phase cells. Figure 1C: Tet21N, a synthetic N-Myc protein inducible expression system on the basis of the tetracycline repressor of *Escherichia coli*, with β-actin serving as loading control. Figure 1D: WAC-2 and SH-SY5Y NB cells.

Figures 2A and 2B. HC-toxin sustainably mediates growth arrest and apoptosis in NB cells with *MYCN* oncogene amplification. Figure 2A: Time course dose-response growth curves of BE(2)-C NB cells cultured with HC-toxin or MeOH solvent control. Figure 2B: Induction of G_0/G_1 cell cycle arrest in the diploid and tetraploid cell fraction of BE(2)-C NB cells by HC-toxin.

Figures 3A and 3B. HC-toxin significantly evokes growth arrest and apoptosis in primary neuroblasts. Figure 3A: Induction of primary neuroblast cell death by HC-toxin (*lower panel*) in a short term culture of stage IV *MYCN* single copy neuroblasts derived from bone marrow (*upper panel*) of a 2 year and 10 months old child compared to MeOH solvent treated controls (*middle panel*). Figure 3B: G_0/G_1 cell cycle arrest and apoptosis mediated by HC-toxin (*lower panels*) in primary *MYCN* single copy neuroblasts (*left upper panel*) derived from the stage I NB of a seven months old infant (*right upper panel*).

Figures 4A-4D. HC-toxin potently induces neuronal differentiation and loss of neuroectodermal stem cell marker expression in NB cells. Figure 4A: Neurite outgrowth in SH-EP and BE(2)-C NB cells following treatment with 15 and 20 nM *Helminthosporium carbonum* (HC)-toxin or MeOH solvent control for 72 h or 14 d, respectively. Figure 4B: Up-regulation of neuronal marker genes on mRNA level. Quantitative „real time“ RT-PCR analysis revealed an induction of neurofilament 3 (*NEF3*, 150 kDa medium), tyrosine hydroxylase (*TH*), microtubule associated protein 2 (*MAP2*), synaptophysin 1 (*SYP1*) and synapsin 1 (*SYN1*) in

HC-toxin treated SH-EP and an induction of the dendritic and synaptic markers in BE(2)-C NB cells compared to respective controls. Figure 4C: Down-regulation of the intermediate filament nestin on mRNA level. Figure 4D: Up-regulation of neuronal markers on protein level. Immunocytochemistry showed an increased expression or induction of neurofilament 3 (anti-Nef 3, 150 kDa medium), tyrosine hydroxylase (anti-TH), microtubule associated protein 2 (anti-MAP2) after 120 h and of synaptophysin 1 (anti-SYP1) and synapsin 1 (anti-SYN1) after 200 h of treatment in SH-EP NB cells, respectively.

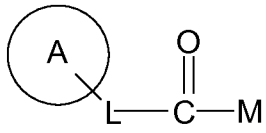
Figures 5A and 5B. HC-toxin strongly reduces anchorage-independent growth and invasive potential of NB cells. Figure 5A: Influence of HC-toxin upon anchorage-independent transformation potential. Pretreated WAC-2 and BE(2)-C NB cells were plated in soft agar (see Example 1). Figure 5B: BE(2)-C (*A1-4*) and WAC-2 (*B1-4*) NB cell invasiveness and extracellular matrix (ECM) degradation was assessed using Boyden chambers. Prior to seeding cells into the upper compartment of the chambers, cells were treated for 5 days with 20 nM HC-toxin or MeOH solvent control. To adjust for differences in proliferation and migratory potential, normalization was obtained using Boyden chambers with control inserts (*A1, A2, B1, B2*) (see Example 1).

Figures 6A and 6B. HC-toxin sustainably activates RB tumor suppression pathways in NB cells. Figure 6A: Activation of RB tumor suppressor function by HC-toxin (HCT) resulting in profound reduction of E2F1 and E2F target genes (Survivin, Mad2, N-myc) as well as in activation of the Skp2-$p27^{kip1}$ pathway in BE(2)-C NB cells. Shown also is the induction of $p21^{cip1/waf-1}$ by HC-toxin. Figure 6B: HC-toxin mediated induction of $p15^{INK4b}$ and $p16^{INK4a}$.

Figures 7A and 7B. HC-toxin promotes no detectable effects upon viability, RB mediated cell cycle control and apoptosis of primary human skin fibroblasts (PHSFs). Figure 7A: Time course dose-response growth curves of PHSFs cultured with HC-toxin or methanol (MeOH) solvent control. Figure 7B: No detectable effect of HC-toxin upon cell cycle, apoptosis (sub-G_1) and granularity (*middle panel*) of PHSFs. Figure 7C: No detectable HC-toxin mediated modulation of RB tumor suppressor activity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention makes use of HDAC inhibitors which are compounds according to the general formula (I)



(I)

The compounds can be tri-, tetra or pentapeptides, preferably tetrapeptides. In the most general meaning classified below under A, the symbols have the following meanings:

A:

A is a 9- to 15-membered heterocycle optionally carrying one or more double bonds, and comprising 3 to 5 units of the type -D-E-G- wherein

D is selected from $-N(R^1)-$, -O-, and $-S(O)(R^2R^3)_m-$; and/or

E is selected from methylene $-CH_2-$, ethylene $-CH_2CH_2-$ and propylene $-CH_2-CH_2-CH_2-$, wherein in the methylene, ethylene and propylene groups one or more H atoms can be replaced by: linear and branched C_1- to C_{10} alkyl groups, which are optionally substituted by one or more substituents from the group -OH, F, -SH, $-SCH_3$, $-NH_2$, $-NHC(NH)NH_2$, $-C(O)OH$, $-C(O)NH_2$, C_6H_5, C_6H_4OH, indolyl, imidazolyl or a salt thereof; and linear and branched C_2- to C_{10} alkylene groups, which are optionally substituted by one or more OH and/or one or more F; or, in case 2 or more even-numbered substituents are present, the named alkyl or alkylene group forms together with a further alkyl or alkylene group on the same or a different carbon atom a 5- to 7-membered cycle; or the named alkyl and alkylene groups form together with the group R^1 a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

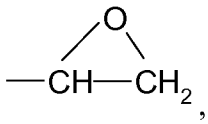
G is a keto group -C(O)- or a thioketo group -C(S)-; and/or

wherein the units -D-E-G- not attached to L can be fully or partially identical with or entirely different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

L is attached to D or E in one unit -D-E- G- and is a linear C_3- to C_5-alkyl group in which one or more carbon atoms may be replaced by non adjacent O atoms, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



hydroxyamino groups –N(OH)H, hydroxymethyl groups $-CH_2OH$, 1-fluoroethyl groups $-CHFCH_3$ and 1-chloroethyl groups $-CHClCH_3$; and/or

R^1 is selected from: H; linear and branched C_1- to C_{10} alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear or branched C_2- to C_{10} alkylene groups which are optionally substituted by one or more OH and/or one or more F; or R^1 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

R^2 and R^3 are independently of each other selected from: H; linear and branched C_1- to C_{10} alkyl groups which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_{10} alkylene groups, which are optionally substituted by one or more OH and/or one or more F; or R^2 and R^3, together with the sulfur atom to which they are attached, form a 5- to 7-membered cycle; or one of R^2, R^3 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

m is 0 or 1,

and/ or a pharmaceutically acceptable salt thereof.

L is always attached to E in one of the units –D-E-G-, which unit has to be different from the other units –D-E-G-.

The cycle A comprises 3, 4 or 5 amino acids. Suitable amino acids are known to the person skilled in the art and comprise natural amino acids and synthetic amino acids. Natural amino acids are those occurring in nature and comprise: glycine, alanine, serine, cysteine, phenylalanine, tyrosine, tryptophane, threonine,

methionine, valine, proline, leucine, isoleucine, lysine, arginine, histidine, aspartic acid, asparagine, glutamic acid, glutamine. The named amino acids are found in the L form in nature. In the context of the present invention, the above referenced natural amino acids in the L-form and in the D-form are preferred.

The double bonds in the cycle A may be a result of enol forms resulting from tautomerism. In case enol forms result from tautomerism between the keto group and a neighbouring -NH or a -CH function, the enol forms may be stabilised or fixed by alkylation. Suitable alkylation methods are known to the person skilled in the art.

The present invention also includes prodrugs of the compounds of formula (I). Examples for suitable prodrugs include molecules according to formula (I) as defined beforehand, and wherein the epoxy function is replaced by an appropriate precursor function therof, e.g. a vicinal diol function, a chlorohydrine function -CH(OH)(Cl)CH- or an olefinic double bond.

Preferred embodiments are classified below under B:

B:

A is a 11- to 13-membered cycle; comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein one or both H atoms can be replaced by: a linear or branched C_1- to C_4 alkyl group, which are optionally substituted by one or more substituents from the group -OH, F, -SH, $-SCH_3$, $-NH_2$, $-NHC(NH)NH_2$, $-C(O)OH$, $-C(O)NH_2$, C_6H_5, C_6H_4OH, indolyl, imidazolyl or a salt thereof; and/or a linear or branched C_2- to C_4 alkylene group, which is optionally substituted by one or more OH and/or one or more F; or the named alkyl and alkylene groups form together with the group R^1 a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

G is a keto group -C(O)-; and/or

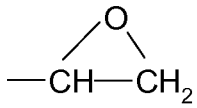
wherein the units -D-E-G- not attached to L can be fully or partially identical with or entirely different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is selected from: H; linear and branched C_1- to C_4 alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_4 alkylene groups which are optionally substituted by one or more OH and/or one or more F; or R^1 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached, and/or

L is attached to E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group in which one or more carbon atoms may be replaced by non adjacent O atoms, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



and hydroxyamino groups –N(OH)H.

The cycle A comprises 4 natural amino acids in the L- or D-form, selected from those cited beforehand.

Even more preferred embodiments are classified below under C:

C:

A is a 12-membered cycle and comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein in one or two units -D-E-G-, one or both H atoms can be replaced by: a linear or branched C_1- to C_4 alkyl group and/or a linear or branched C_2- to C_4 alkylene group; and wherein in only one unit -D-E-G-, one H atom in $-CH_2-$ is replaced by a linear or branched C_1- to C_4 alkyl group or a linear or branched C_2- to C_4 alkylene group, with the said alkyl or alkylene group forming together with R^1 in the neighbouring NR^1 group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

G is a keto group -C(O)-; and/or

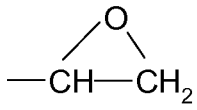
at least 2 units -D-E-G- are different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is selected from: H; linear and branched C_1- to C_4 alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_4 alkylene groups, which are optionally substituted by one or more OH and/or one or more F; and wherein in exactly one unit -D-E-G-, R^1 forms together with the said alkyl or alkylene in the neighbouring $-CH_2-$ group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

L is attached to E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



and hydroxyamino groups –N(OH)H; and/or

the cycle A comprises 4 natural amino acids in the L- or D-form.

Embodiments, which are still preferred over those under C, are classified below under D:

D:

A is a 12-membered cycle and comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein in one or two units -D-E-G-, one or both H atoms can be replaced by: a methyl or ethyl group; and wherein in only one unit -D-E-G-, one H

atom in -CH_2- is replaced by a linear or branched C_1- to C_4 alkyl group or a linear or branched C_2- to C_4 alkylene group, and wherein the said alkyl or alkylene group forms together with R^1 in the neighbouring NR^1 group a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached;

G is a keto group -C(O)-; and/or

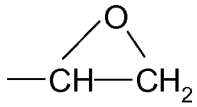
wherein at least 2 units -D-E-G- are different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is H or a linear or branched C_1- to C_4-alkyl group in three of the units -D-E-G-; and wherein in the other unit –D-E-G, R^1 forms together with the said alkyl or alkylene in the neighbouring -CH_2- group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

L is attached to E in one unit -D-E-G- and is a C_3- to C_5-alkyl group, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups

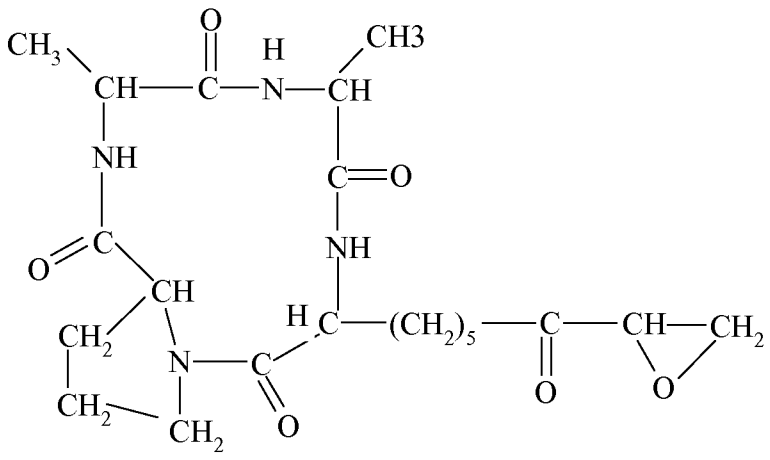


and hydroxyamino groups –N(OH)H; and/or

the cycle A comprises 4 natural amino acids in the L- or D-form.

Within the definitions made beforehand for embodiments A, B, C and D, the individual meanings for each substituent or each group are not construed to be restricted to the embodiment under which it is mentioned, i.e. a definition cited under C my be combined with the definitions made under e.g. A, B, or D. The entire combination of all the definitions for each substituent made under each of A, B, C and D is however preferred.

As an example, for the compounds according to the general formula (I), reference is made to formula (II), i.e. *Helminthosporium carbonum* toxin (HC-Toxin).



(II)

In preferred embodiment, the present invention demonstrates that *Helminthosporium carbonum* (HC)-toxin, an inhibitor of histone deacetylases (HDACs), induces G_0/G_1 cell cycle arrest, blockage of DNA-replication and apoptosis in neuroblastoma cell lines with and without *MYCN* oncogene amplification, as well as in primary neuroblasts. Only nanomolar concentrations of HC-toxin are required to achieve this result. Cell cycle exit is followed by neuronal differentiation and loss of neuroectodermal stem cell marker expression as well as by decrease in anchorage-independent plating efficiency and invasiveness *in vitro*.

Neuroblastoma (NB) cell maturation is linked to activation of retinoblastoma (RB) tumor suppressor function resulting in profoundly reduced expression of E2F target genes as well as in activation of the Skp2-$p27^{kip1}$ pathway. Moreover, $p21^{cip1/waf-1}$/ RB, $p15^{INK4b}$/ RB and $p16^{INK4a}$/ RB mediated cell cycle control is enforced by HC-toxin. As a result, conversion of NB cells from malignant towards more benign by

nanomolar doses of the HDAC inhibitor *Helminthosporium carbonum* toxin is associated with activation of RB tumor suppression pathways. These effects seem tumor cell selective as HC-toxin promotes no detectable effects upon cell cycle, viability and RB activity of primary human skin fibroblasts (PHSFs).

The present inventors elucidated the potential of the HDAC inhibitor (HDACI) *Helminthosporium carbonum* (HC)-toxin (20) to suppress malignant qualities of NB cells. NB cell lines and primary neuroblasts were treated with nanomolar doses of HC-toxin. Proliferation assays and flow cytometric analysis were performed to analyze the impact upon cell growth, cell cycle and apoptosis. The induction of neuronal differentiation by HC-toxin was elucidated by quantitative real time RT-PCR and immunocytochemistry. The invasive potential of neuroblasts as well as their capacity to grow in anchorage-independent manner following HC-toxin treatment were assessed by *in vitro* cell transformation and invasion assays. These experiments showed that HC-toxin converses in nanomolar dosage NB cells from malignant towards more benign.

RB controlled tumor suppression pathways are known to be disabled in numerous human cancers (21). Deregulation of RB signalling pathways such as the RB-E2F and the RB-Skp2-$p27^{kip1}$ pathways causes impairment of cell cycle exit and thus leads to uncontrolled cell cycle transition (21, 55).

In neuroblastoma, a distinct correlation was identified between poor patient prognosis and deregulated E2F activity as well as high expression of the mitotic checkpoint protein and E2F target Mad2 (22). Aberrant *MAD2* transcript levels were found to be linked to high *E2F1* messenger RNA levels in a near-linear correlation suggesting RB pathway alterations in NB with poor prognosis (22). Moreover, Westermann and colleagues identified a clear link between progressing NB and an impaired Skp2-$p27^{kip1}$ pathway (unpublished data). In benign NB, this pathway was found to be functional.

Therefore, the present inventors hypothesized that conversion of NB cells from malignant towards more benign by nanomolar doses of the HDACI HC-toxin is associated with activation of RB tumor suppression pathways. Western analysis was applied to assess the influence of HC-toxin upon RB tumor suppression signalling pathways in four *MYCN* amplified NB cell lines. These experiments showed that conversion of NB cells from malignant towards more benign by HC-toxin is associated with activation of RB tumor suppression pathways.

Out of a panel of diverse small- molecule HDACIs covering all chemical classes, HC-toxin was identified in screening experiments to elicit efficiently and with high potency cell cycle arrest and apoptosis in five NB cell lines harboring different *MYCN* oncogene expression. Growth curve analysis comparing HC-toxin treated versus untreated NB cells profoundly differed with only little dividing activity remaining in HC-toxin treated NB cell populations. Similar results were obtained when treating primary neuroblasts. Cell cycle analysis revealed a cell cycle arrest in G_0/G_1 as well as apoptosis both in NB cell lines and in primary neuroblasts treated with HC-toxin. Following regain of cell cycle control, induction of neuronal differentiation and down-regulation of the transcript of the neuroectodermal stem cell marker nestin was observed in HC-toxin treated NB cells. Furthermore, HC-toxin strongly decreased anchorage-independent growth potential and invasive capacity of NB cells. Taken together, the HDAC inhibitor HC-toxin reverses in nanomolar dosage NB cells from malignant towards more benign.

The present inventors also surmised that suppression of malignant qualities of NB cells by HC-toxin is associated with activation of RB tumor suppression pathways. Inhibition of HDACs by 10 to 20 nM doses of HC-toxin was found to enhance the growth inhibitory function of RB and its control of E2F dependent effectors as well as the Skp2-$p27^{kip1}$ pathway in four *MYCN*-amplified NB cells. Moreover, $p21^{cip1/waf-1}$/ RB, $p15^{INK4b}$/ RB and $p16^{INK4a}$/ RB mediated cell cycle control was enforced by HC-toxin. In conclusion, reversion of NB cells from malignant towards more benign by HC-toxin is associated with activation of RB tumor suppression pathways.

Cell cycle, viability and RB activity of PHSFs remained unaltered under HC-toxin treatment. HC-toxin accordingly imposed no unfavorable effects upon these untransformed dividing cells. However, *in vivo* animal testing is imperative in respect to the further evaluation of HC-toxin mediated toxicity upon normal tissues as well as in respect to its bioavailability.

In conclusion, induction of cycle arrest, apoptosis and neuronal differentiation in NB cells by the HDACI *Helminthosporium carbonum* toxin is associated with activation of RB tumor suppression pathways *in vitro*. Suppression of malignancy by pharmacologic activation of RB tumor suppression pathways presents a novel approach to NB therapy.

It is understood that the present invention is not limited to the particular methodology, protocols, vectors, and reagents, etc., described herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a gene" is a reference to one or more genes and includes equivalents thereof known to those skilled in the art and so forth. Indeed, one skilled in the art can use the methods described herein to express any native gene (known presently or subsequently) in plant host systems.

The present invention uses terms and phrases that are well known to those in the art of molecular biology. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein, and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described herein are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, microbial culture, cell culture, tissue culture, transformation, transfection, transduction, analytical chemistry, organic synthetic chemistry, chemical syntheses, chemical analysis, and pharmaceutical formulation and delivery. Generally, enzymatic reactions and purification and/or isolation steps are performed according to the manufacturers' specifications. The techniques and procedures are generally performed according to conventional methodology (Molecular Cloning, A Laboratory Manual, 3rd. edition, edited by Sambrook & Russel Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001).

The present invention includes all stereoisomeric forms of the compounds of the formula (I). Centers of asymmetry that are present in the compounds of formula (I) all independently of one another have S configuration or R configuration. The invention includes all possible enantiomers and diastereomers and mixtures of two or more stereoisomers, for example mixtures of enantiomers and/or diastereomers, in all ratios. Thus, compounds according to the present invention which can exist as enantiomers can be present in enantiomerically pure form, both as levorotatory and as dextrorotatory antipodes, in the form of racemates and in the form of mixtures of the two enantiomers in all ratios. In the case of a cis/trans isomerism the invention

includes both the cis form and the trans form as well as mixtures of these forms in all ratios. All these forms are an object of the present invention. The preparation of individual stereoisomers can be carried out, if desired, by separation of a mixture by customary methods, for example by chromatography or crystallization, by the use of stereochemically uniform starting materials for the synthesis or by stereoselective synthesis. Optionally a derivatization can be carried out before a separation of stereoisomers. The separation of a mixture of stereoisomers can be carried out at the stage of the compounds of the formula (I) or at the stage of an intermediate during the synthesis. The present invention also includes all tautomeric forms of the compounds of formula (I).

In case the compounds according to formula (I) contain one or more acidic or basic groups, the invention also comprises their corresponding pharmaceutically or toxicologically acceptable salts, in particular their pharmaceutically utilizable salts. Thus, the compounds of the formula (I), which contain acidic groups, can be present on these groups and can be used according to the invention, for example, as alkali metal salts, alkaline earth metal salts or as ammonium salts. More precise examples of such salts include sodium salts, potassium salts, calcium salts, magnesium salts or salts with ammonia or organic amines such as, for example, ethylamine, ethanolamine, triethanolamine or amino acids. Compounds of the formula (I), which contain one or more basic groups, i.e. groups which can be protonated, can be present and can be used according to the invention in the form of their addition salts with inorganic or organic acids. Examples for suitable acids include hydrogen chloride, hydrogen bromide, phosphoric acid, sulfuric acid, nitric acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acids, oxalic acid, acetic acid, tartaric acid, lactic acid, salicylic acid, benzoic acid, formic acid, propionic acid, pivalic acid, diethylacetic acid, malonic acid, succinic acid, pimelic acid, fumaric acid, maleic acid, malic acid, sulfaminic acid, phenylpropionic acid, gluconic acid, ascorbic acid, isonicotinic acid, citric acid, adipic acid, and other acids known to the person skilled in the art. If the compounds of the formula (I) simultaneously contain acidic and basic groups in the molecule, the invention also includes, in addition to the salt forms mentioned, inner salts or betaines (zwitterions). The respective salts according to the formula (I) can be obtained by customary methods, which are known to the person skilled in the art like, for example by contacting these with an organic or inorganic acid or base in a solvent or dispersant, or by anion exchange or cation exchange with other salts. The present invention also includes all salts of the compounds of the formula (I), which, owing to low physiological compatibility, are not directly suitable for use in

pharmaceuticals but which can be used, for example, as intermediates for chemical reactions or for the preparation of pharmaceutically acceptable salts.

The present invention furthermore includes all solvates of compounds of the formula (I), for example hydrates or adducts with alcohols, active metabolites of the compounds of the formula (II), and also derivatives and prodrugs of the compounds of the formula (I) which contain physiologically tolerable and cleavable groups, for example esters, amides and compounds in which the N-H group depicted in formula (I) is replaced with an N-alkyl group, such as N-methyl, or with an N-acyl group, such as N-acetyl or N-argininyl, including pharmaceutically acceptable salts formed on functional groups present in the N-acyl group.

The compounds according to general formula (I) and their precursors can be prepared according to methods published in the literature or, respectively, analogous methods. Appropriate methods have been published in, for example, Houben-Weyl, Methoden der Organischen Chemie (Methods of Organic Chemistry), Thieme-Verlag, Stuttgart, or Organic Reactions, John Wiley & Sons, New York.

All reactions for the synthesis of the compounds of the formula (I) are per se well-known to the skilled person and can be carried out under standard conditions according to or analogously to procedures described in the literature, for example in Houben-Weyl, Methoden der Organischen Chemie (Methods of Organic Chemistry), Thieme-Verlag, Stuttgart, or Organic Reactions, John Wiley & Sons, New York. Depending on the circumstances of the individual case, in order to avoid side reactions during the synthesis of a compound of the formula (I), it can be necessary or advantageous to temporarily block functional groups by introducing protective groups and to deprotect them in a later stage of the synthesis, or introduce functional groups in the form of precursor groups which in a later reaction step are converted into the desired functional groups. Such synthesis strategies and protective groups and precursor groups, which are suitable in an individual case, are known to the skilled person. If desired, the compounds of the formula (I) can be purified by customary purification procedures, for example by recrystallization or chromatography. The starting compounds for the preparation of the compounds of the formula (I) are commercially available or can be prepared according to or analogously to literature procedures.

The compounds according to the formula (I) can also be used in combination with other pharmaceutically active compounds, preferably compounds which are able to enhance the effect of the compounds according to the general formula (I). Examples of such compounds include: (i) antimetabolites, cytarabine, fludarabine, 5-fluoro-2′-deoxyuridine, gemcitabine, hydroxyurea or methotrexate; (ii) DNA-fragmenting agents, bleomycin, (iii) DNA-crosslinking and alkylating agents, chlorambucil, cisplatin, carboplatin, fotemustine, cyclophosphamide, ifosfamide, dacarbazine or nitrogen mustard; (iv) intercalating agents, adriamycin (doxorubicin) or mitoxantrone; (v) protein synthesis inhibitors, L-asparaginase, cycloheximide, puromycin or diphteria toxin; (vi) topoisomerase I poisons, camptothecin or topotecan; (vii) topoisomerase II poisons, etoposide (VP-16) or teniposide; (viii) microtubule-directed agents, colcemid, colchicine, paclitaxel (taxol), docetaxel (taxotere), vinblastine or vincristine; (ix) kinase inhibitors, flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); (x) miscellaneous investigational agents, trichostatin A, thioplatin, PS-341, phenylbutyrate, ET-18-OCH_3, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols, quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; (xi) hormones, glucocorticoids or fenretinide; (xii) hormone antagonists, tamoxifen, finasteride or LHRH antagonists, (xiii) demethylating agents, 5-azacytidine, 5-aza-2'deoxycytidine, 5,6-dihydro-5-azacytidine, or (xiv) a combination of any of the pharmaceuticals given above or use in high-dose chemotherapy regimens including stem cell transplantation; (xv) differentiation inducing agents such as retinoic acid derivatives; (xvi) ionizing radiation therapy, MIBG-therapy and conventional radiation therapy.

The compounds of the formula (I) and their pharmaceutically acceptable salts, optionally in combination with other pharmaceutically active compounds, can be administered to animals, preferably to mammals, and in particular to humans, as pharmaceuticals by themselves, in mixtures with one another or in the form of pharmaceutical preparations. Further subjects of the present invention therefore also are the compounds of the formula (I) and their pharmaceutically acceptable salts for use as pharmaceuticals, their use as inhibitors of DNMTs and/or DNA methylation, and in particular their use in the therapy and prophylaxis of the above-mentioned syndromes as well as their use for preparing pharmaceuticals for these purposes. Furthermore, subjects of the present invention are pharmaceutical preparations (or pharmaceutical compositions), which comprise an effective dose of at least one compound of the formula (I) and/or a pharmaceutically acceptable

salt thereof and a pharmaceutically acceptable carrier, i.e. one or more pharmaceutically acceptable carrier substances and/or additives.

The pharmaceuticals according to the invention can be administered orally, for example in the form of pills, tablets, lacquered tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, aqueous, alcoholic or oily solutions, syrups, emulsions or suspensions, or rectally, for example in the form of suppositories. Administration can also be carried out parenterally, for example subcutaneously, intramuscularly or intravenously in the form of solutions for injection or infusion. Other suitable administration forms are, for example, percutaneous or topical administration, for example in the form of ointments, tinctures, sprays or transdermal therapeutic systems, or the inhalative administration in the form of nasal sprays or aerosol mixtures, or, for example, microcapsules, implants or rods. The preferred administration form depends, for example, on the disease to be treated and on its severity.

The preparation of the pharmaceutical preparations can be carried out in a manner known per se. To this end, one or more compounds of the formula (I) and/or their pharmaceutically acceptable salts, together with one or more solid or liquid pharmaceutical carrier substances and/or additives (or auxiliary substances) and, if desired, in combination with other pharmaceutically active compounds having therapeutic or prophylactic action, are brought into a suitable administration form or dosage form, which can then be used as a pharmaceutical in human or veterinary medicine.

For the production of pills, tablets, sugar-coated tablets and hard gelatin capsules it is possible to use, for example, lactose, starch, for example maize starch, or starch derivatives, talc, stearic acid or its salts, etc. Carriers for soft gelatin capsules and suppositories are, for example, fats, waxes, semisolid and liquid polyols, natural or hardened oils, etc. Suitable carriers for the preparation of solutions, for example of solutions for injection, or of emulsions or syrups are, for example, water, physiologically sodium chloride solution, alcohols such as ethanol, glycerol, polyols, sucrose, invert sugar, glucose, mannitol, vegetable oils, etc. It is also possible to lyophilize the compounds of the formula (I) and their pharmaceutically acceptable salts and to use the resulting lyophilisates, for example, for preparing preparations for injection or infusion. Suitable carriers for microcapsules, implants or rods are, for example, copolymers of glycolic acid and lactic acid.

Besides the compound or compounds according to the invention and carriers, the pharmaceutical preparations can also contain additives, for example fillers, disintegrants, binders, lubricants, wetting agents, stabilizers, emulsifiers, dispersants, preservatives, sweeteners, colorants, flavorings, aromatizers, thickeners, diluents, buffer substances, solvents, solubilizers, agents for achieving a depot effect, salts for altering the osmotic pressure, coating agents or antioxidants.

The dosage of the compound of the formula (I) to be administered and/or of a pharmaceutically acceptable salt thereof depends on the individual case and is, as is customary, to be adapted to the individual circumstances to achieve an optimum effect. Thus, it depends on the nature and the severity of the disorder to be treated, and also on the sex, age, weight and individual responsiveness of the human or animal to be treated, on the efficacy and duration of action of the compounds used, on whether the therapy is acute or chronic or prophylactic, or on whether other active compounds are administered in addition to compounds of the formula (I). The daily dose can be administered in a single dose or, in particular when larger amounts are administered, be divided into several, for example two, three or four individual doses. In some cases, depending on the individual response, it may be necessary to deviate upwards or downwards from the given daily dose.

The following examples are given to illustrate the present invention. It should be understood, however, that the spirit and scope of the invention is not to be limited to the specific conditions or details described in these examples but should only be limited by the scope of the claims that follow. All references identified herein, including U.S. patents, are hereby expressly incorporated by reference.

EXAMPLES

Example 1

Materials and Methods

1. Cell Culture.

Human NB cell lines with (BE(2)-C, Kelly, LA-N-1 and NGP) and without (SH-EP, SH-SY5Y) *MYCN* oncogene amplification were cultured according to the depositor's instructions. Tet21N NB cells engineered to variably express

tetracycline-controlled N-myc protein and WAC-2 NB cells containing a transfected functional human *MYCN* oncogene governing enhanced expression were cultured as previously described (23, 24). PHSFs derived from an adult undergoing surgical treatment were grown in DMEM supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) l-glutamine.

2. Patients and Samples.

NB tumor and bone marrow specimens were obtained from patients as part of their routine care following informed consent. Primary neuroblasts (*MYCN* single copy) isolated from a stage 1 NB of a seven months old infant were grown in DMEM augmented with 1% autologous serum. Stage IV neuroblasts (*MYCN* single copy) derived from bone marrow metastasis of a 2 year and 10 months old child were isolated by Ficoll-paque (Amersham Biosciences, Buckinghamshire, UK).

3. Differentiation.

Cells were treated for the indicated periods of time with indicated concentrations of HC-toxin (Sigma-Aldrich, Schnelldorf, Germany; Lot # 054K4121) or methanol (MeOH; Roth, Karlsruhe, Germany) as solvent control (% v/v).

4. Real Time Reverse Transcription Polymerase Chain Reaction (real time RT-PCR).

Relative quantification of *NEF3, NES, MAP2, SYN1, SYP1* and *TH* mRNA expression was carried out by real time RT-PCR with SYBR Green Dye using an ABI Prism 7000 thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA). Total RNA was isolated from cells with the RNeasy kit from Qiagen with an on-column DNAse digestion (Qiagen, Hilden, Germany) incorporated. Reverse transcription (RT)-PCR was performed with moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers (Invitrogen, Carlsbad, CA) in a volume of 40 µl based upon 1 µg of total RNA per reaction. The RNA was primed at 60° C for 10 min, subjected to transcription at 37° C for 1 h and finally heated up to 65° C for 10 min. Real time RT-PCR was implemented in a reaction volume of 25 µl containing 1 x qPCR MasterMix and SYBR Green I Dye (SYBR Green I kit, Eurogentec, Seraing, Belgium), appropriate amounts of first strand complementary (c)-DNA as template and specific primers selected from Qiagen's QuantiTect Primer Assay program (*GAPD*, # QT00079247;

NEF3, # QT00073885; *NES*, # QT01015301; *MAP2,* # QT00057358; *SYN1*, # QT00045913; *SYP1*, # QT00013062; *TH*, # QT00067221) or purchased from Thermohybaid (Ulm, Germany): *ACTB* (forward) 5'-GCATCCCCCAAAG-TTCACAA-3'; *ACTB* (reverse) 5'-AGGACTGGGCCATTCTCCTT-3'; *ASH2L* (forward) 5'-GTTTTGTTAGCCCTACATGATCGA-3'; *ASH2L* (reverse) 5'-GAGTAGCCCTTCTCTCC- AACCA-3', *HPGD* (forward) 5'-GAGGTGAAGGCGGCATCAT-3'; and *HPGD* (reverse) 5'-CCAACTATGCCATGCTTTGAAG-3'. Thermal cycling was conducted under standard conditions. Each sample was analyzed at least in duplicate with high reproducibility. Specificity of resulting amplicons was confirmed by melting curves and agarose gel electrophoresis. C_t-values of target genes were normalized with corresponding C_t-value means of the reference genes *ACTB*, *GAPD*, *ASH2L* and *HPGD*, which remained unaltered under all conditions investigated.

5. Western Analysis.

Whole cell lysates were subjected to Western analysis as previously described (25) using antibodies directed against E2F1 (Santa Cruz Biotechnologies, Santa Cruz, California), H4 pan Ac (Upstate, Lake Placid, NY), Mad2 (Becton Dickinson, Bedford, MA), N-myc (Becton Dickinson, Bedford, MA),), $p15^{INK4b}$ (Dianova, Hamburg, Germany), $p16^{INK4A}$ (Dianova, Hamburg, Germany), $p21^{cip1/waf-1}$ (Upstate, Lake Placid, NY), $p27^{kip1}$(Dianova, Hamburg, Germany), RB (Becton Dickinson, Bedford, MA), Skp2 (Zymed Laboratories, Invitrogen Immunodetection, San Francisco, CA) and Survivin (Santa Cruz Biotechnologies, Santa Cruz, California). Equivalent protein loading was assessed by immunodetection of β-actin (Dianova, Hamburg, Germany).

6. Immunofluorescence.

Immunofluorescence was performed on cells grown on poly-D-lysine coated glass coverslips placed in 24-well chamber slides (Nunc, Roskilde, Denmark), fixed with 2 % paraformaldehyde (Merck) in 0.1 M PO_4 buffer and subsequently permeabilized with phosphate buffered saline containing 0.1 % Triton X-100 (Serva) (PBST). Unspecific labeling was blocked with a solution of 10 % normal goat serum, 0.25 % bovine serum albumin dissolved in PBST. Cells were incubated for 12 h at 4° C with 1:500 dilutions of the primary antibodies rabbit anti-Nef 150 kD (Chemicon, Temecula, CA), mouse anti-Map2 (Chemicon), mouse anti-Syp 1 (Synaptic Systems, Goettingen, Germany), mouse anti-Syn1 (Synaptic Systems) and rabbit anti-Th (Chemicon) dissolved in blocking buffer. Cy3-conjugated secondary antibodies goat anti-mouse (Jackson ImmunoResearch, Baltimore, PA) and goat anti-rabbit (Rockland, Gilbertsville, PA) were applied as 1:100 dilutions in PBST for 3 hrs at room temperate. After washing with PBS, cell-containing coverslips were placed upside down on microscope slides covered with PBS/Glycerol 1:1. Fluorescent images shown in Figure 2 b were acquired using a Leica confocal microscope (Leica Microsystems, Wetzlar, Germany; Model TCS SP2 with AOBS) including standard software used for data acquisition provided by Leica.

7. Cell Proliferation Assay.

To perform growth curve analysis, cells were plated, treated with different concentrations of HC-toxin or solvent control (%v/v), harvested on the indicated day and evaluated for number and viability by trypan blue (Sigma-Aldrich, Schnelldorf, Germany) exclusion.

8. Flow Cytometry.

Flow cytometric analysis was performed using a Galaxy Pro Flow Cytometer (Partec, Muenster, Germany). Natively sampled cells were prepared in 2.1% citric acid/ 0.5% tween 20 (26) and stained with 50µg/ml DAPI dissolved in phosphate buffer (7.2 g Na_2HPO_4 x $2H_2O$ in 100ml distilled water, pH 8.0). DNA-index and cell cycle measurements are based upon the analysis of 30.000 – 100.000 cells. For histogram analyses acquired in the FCS-mode, the Multicycle program (Phoenix Flow Systems, San Diego, CA) was applied. Human lymphocytes from healthy

donors served as internal standard for calibrating the diploid DNA-index. The mean coefficient of variations (CV) of these measurements was 0.9.

Living, dead and apoptotic cell fractions were evaluated using a FACS-Calibur (Becton Dickinson, Heidelberg, Germany). Cells were prepared according to Nicoletti (27) and cell stain measurements were acquired in Fl-2 in logarithmic mode. Apoptotic cell fractions were calculated by setting the gate over the first three decades. Cell Quest software (Becton Dickinson, Heidelberg, Germany) was utilized for histogram and plot analyses. Each histogram and dot plot represents 10.000 cells.

9. Cell Transformation Assay.

Cells were seeded into soft agar according to Chemicon´s Cell Transformation Assay and treated with solvent (MeOH) or HC-toxin. Only colonies stained with cell stain solution were counted. The experiment was independently carried out three times in duplicates. Student's *t* test was applied to assess differences between treatment conditions.

10. Motility Invasion Assay.

In vitro cell invasion was evaluated in Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA) according to the method of Albini *et al.* (28). To adjust for differences in proliferation and migratory potential, normalization was obtained using Boyden chambers with control inserts. NB cells invading the Matrigel or only passing through the pores and attaching to the lower surface of the membrane were microscopically visualized following fixation in 100 % MeOH and staining with Giemsa. Cell invasivity is expressed as the percentage of cells invading through the Matrigel coated membrane compared to the number of cells (100%) migrating through the uncoated membrane. Nuclei were counted at 200 x magnification. 10 representative fields per membrane were analyzed. Assays were performed three times. Student's *t* test was used to examine differences between treatment conditions.

Example 2

Growth arrest and apoptosis in NB cells with *MYCN*-single copy status and with transfected human *MYCN* oncogene governing enhanced expression.

The present inventors systematically examined a panel of small-molecule HDACIs covering the chemical classes of carboxylates, hydroxamic acid derivatives, benzamides and tetracyclic peptides for their efficacy and potency to elicit anti-tumoral effects in experimental NB. The NB cell lines SH-EP, SH-SY5Y, WAC-2 and BE(2)-C as well as Tet21N NB cells engineered to variably express tetracycline-controlled N-myc protein were used in these screening experiments (data not shown). The cyclic tetrapeptide HC-toxin isolated from the fungus *Helminthosporium carbonum* was identified to elicit the strongest effects regarding growth arrest and apoptosis as well as neuronal differentiation and loss of neuroectodermal stem cell marker expression at the lowest concentration in 10 to 20 nanomolar range.

The present inventors analyzed the effect of HC-toxin upon NB cell proliferation after proving a significant increase in histone 4 acetylation in SH-EP NB cells after 6 h of 10 – 20 nM HC-toxin exposure (Fig. 1*A*, *upper panel*). Cells were treated with various concentrations of HC-toxin (HCT) and MeOH solvent control (% v/v) for 6 h or remained untreated (ut). Proteins were extracted and analyzed for panAc-H4 by Western analysis with Coomassie stain of the gel serving as loading control. We first studied the number of viable SH-EP cells and found a clear dose-dependent decrease in viable cells over time by HC-toxin treatment (Fig. 1*A*). Cell cycle analysis revealed a significant G_0/G_1 arrest from 24 h to 72 h of HC-toxin treatment (Fig. 1*B*). Moreover, a strong induction of apoptosis was observed at 48 h and 72 h of HC-toxin treatment (Fig. 1*B*). With respect to Fig. 1*B*, changes in phases of the cell cycle and in apoptosis were analyzed as described in Example 1. After 72 h HC-toxin treatment there was an increase in SH-EP NB cell granularity (Fig. 1B, *middle panel*). In parallel, a profound increase in SH-EP cell granularity was observed (Fig. 1*B*).

To elucidate the effect of HC-toxin upon NB cells harboring enhanced *MYCN*-oncogene expression, the synthetic inducible expression system Tet21N on the basis of the tetracycline repressor of *Escherichia coli* was used (23). Western analysis denotes change in N-myc protein expression dependent upon tetracycline concentration, showing barely detectable N-myc protein expression in the presence and high expression in the absence of tetracycline corresponding to a 50 fold difference in *MYCN* expression on mRNA level (23) (Fig. 1*C, upper panel*). Analysis of the number of viable cells under HC-toxin treatment detected a statistically significant stronger reduction in proliferation of N-myc protein expressing Tet21N cells compared to those lacking N-myc expression suggesting

that HC-toxin potently antagonizes N- myc mediated NB cell aggressiveness (Fig. 1*C, lower panel*). This is corroborated by the distinct inhibition of WAC-2 NB cell proliferation by HC-toxin despite approximately 100 x higher *MYCN* mRNA expression compared to the parental cell line SH-EP (24) (Fig. 1*D, left panel*). With respect to Fig. 1D, harvested cells were evaluated for number and viability by trypan blue exclusion. Results presented are means ± SDs of three independent experiments performed in triplicates. SH-SY5Y cells, subcloned from the same NB tumor as SH-EP (29) but characterized by a neuronal phenotype, are also sensitive to inhibition of proliferation by nanomolar HC-toxin treatment (Fig. 1*D, right panel*). In summary, HC-toxin mediates significant inhibition of proliferation at nanomolar concentration in a panel of NB cell lines independent of their phenotype and with particular efficacy against NB cells harboring enhanced *MYCN* oncogene expression.

Example 3

Growth arrest and apoptosis in NB cells with *MYCN* oncogene amplification.

The physiologically *MYCN* amplified NB cell line BE(2)-C consisting of a diploid and a tetraploid cell fraction was chosen for analysis. HC-toxin evoked in 10 – 20 nM dosage an increase in histone 4 acetylation after 6 h (Fig. 2*A*, *upper panel*). With respect to Fig. 2*A*, harvested cells were evaluated for number and viability by trypan blue exclusion. Results presented are means ± SDs of three independent experiments performed in triplicates. Induction of histone 4 acetylation (panAc-H4) by HC-toxin is given in the *upper panel*. Cells were treated with various concentrations of HC-toxin (HCT) and MeOH solvent control (% v/v) for 6 h or remained untreated (ut). Proteins were extracted and analyzed for panAc-H4 by Western analysis with Coomassie stain of the gel serving as loading control. Proliferation assays and flow cytometric analysis revealed a potent inhibition of cell proliferation (Fig. 2*A*), apoptosis (data not shown) as well as a significant G_0/G_1 cell cycle arrest in both cell fractions induced by HC-toxin (Fig. 2*B*). An overall reduction in tetraploid cells was observed upon HC-toxin treatment (Fig. 2*B*). With respect to Fig. 2*B*, cells were treated with different concentrations of HC-toxin or MeOH solvent control for indicated time periods. Changes in phases of the cell cycle were analyzed as described in Example 1. Shown *at right* are exemplarily FACS profiles of solvent control and HC-toxin treated cells after 72 h of exposure. Striped areas represent S-phase cells. These findings correspond to results obtained in the SH-EP NB model.

Example 4

Growth arrest and apoptosis in primary neuroblasts.

Because cell lines grown in culture might not reflect primary NBs, we exposed primary neuroblasts to HC-toxin. Stage IV neuroblasts metastasized to bone marrow (Fig. 3*A*, *upper panel*) were apoptotic after 48 h of treatment (Fig. 3*A, lower panel*) compared to controls (Fig. 3*A, middle panel*). HC-toxin treatment of stage I neuroblasts (Fig. 3*B*, *left upper panel*) derived from the NB tumor of a 7 months old infant (Fig. 3*B*, *right upper panel*) resulted in a significant G_0/G_1 arrest and the induction of apoptosis (Fig. 3*B*, *lower panels*). With respect to Fig. 3*B*, differences compared to MeOH solvent treated controls reached statistical significance (*right lower panel*) with $P < .05$ (*). In conclusion, HC-toxin has similar effects in established NB cell lines and in primary NB cultures.

Example 5

Neuronal differentiation and loss of neuroectodermal stem cell marker expression.

Microscopic analysis of HC-toxin treated NB cells revealed dose-dependently - apart from structural alterations specific for apoptotic cells - extensive changes in morphology towards a neuronal phenotype. This was characterized by rounded cell bodies, intermediate to long-length fine neurites and extensive interconnections among neurites (Fig. 4*A*). With respect to Fig. 4*A*, representative sections were photographed under an inverted phase-contrast microscope (Olympus, 200 x; scale bar: 15 µm). Some NB cell lines including BE(2)-C responded to HC-toxin treatment with ganglionic-like formations (Fig 4*A*, *right lower panel*). Approximately 90 – 95 % of HC-toxin treated SH-EP cells (Fig 4*A*, right *upper panel*) exhibited neuronal differentiation after 72 h of treatment whereas first effects in BE(2)-C cells were observed after 96 h of exposure reaching their maximum after 14 d with interconnections among neurites and pseudoganglionic formations.

Expression of neuronal markers was evaluated on mRNA and protein level to further characterize the morphologic changes. Quantitative real time RT-PCR analysis revealed a up-regulation of the axonal marker neurofilament 3, 150 kDa (*NEF3*), the rate limiting enzyme of catecholamine synthesis in adrenergic neurons,

tyrosine hydroxylase (*TH*), and of the dendritic marker microtubule associated protein 2 (*MAP2*) in SH-EP NB cells after 72 h of HC-toxin treatment and of the synaptic markers synaptophysin 1 (*SYP1*) and synapsin 1 (*SYP1*) after 120 h of treatment (Fig. 4*B, left graph*). In BE(2)-C NB cells, the dendritic and synaptic marker gene levels were found to be higher in HC-toxin treated NB cells (Fig. 4*B, right graph*). Immunocytochemistry, exemplarily demonstrated for SH-EP NB cells (Fig. 4*D*), uncovered parallel to the mRNA findings an increase in Nef3, Th, Map2, Syp1 and Syn1 protein expression in HC-toxin treated cells compared to controls.

The intermediate filament nestin is expressed in multipotent neuroectodermal precursor cells and is down-regulated as these cells divide and differentiate along their respective neural or glial lineages (30). In highly metastatic glioblastomas and astrocytomas, infiltrating cells are characterized by elevated nestin levels (31, 32). Also, it has been proposed that nestin is one mediator of N-myc associated aggressiveness in neuroblastoma (33). We therefore investigated the influence of HC-toxin upon nestin mRNA levels in BE(2)-C NB cells belonging to the most malignant NB cell type (34). Nestin mRNA expression was significantly down-regulated in HC-toxin treated BE(2)-C cells (Fig. 4*C*). Quantitative "real time" RT-PCR analysis disclosed a strong decrease in mRNA amounts of the neuroectodermal stem cell marker in HC-toxin treated BE(2)-C NB cells compared to controls. In conclusion, the HDACI HC-toxin induces neuronal differentiation and loss of neuroectodermal stem cell marker expression at nanomolar concentration in NB cell lines.

Example 6

Loss of anchorage-independent growth and invasive potential of NB cells.

Anchorage-independent growth potential and *in vitro* invasiveness were evaluated following treatment of BE(2)-C and WAC-2 cells with 20 nM HC-toxin or MeOH solvent control for 72 h. Analysis revealed that HC-toxin treated BE(2)-C and WAC-2 cells are characterized by a dose-dependent significant loss of anchorage-independent growth potential (Fig. 5*A*) and of *in vitro* invasiveness in Boyden chambers (Fig. 5*B*). With respect to Fig. 5*A*, after 12 and 24 days, respectively, plates were photographed and colonies stained with cell stain solution were counted. The experiment was repeated independently three times in duplicates with consistent results. The mean numbers of colonies for each cell line and SDs are

presented in the bar graphs shown at *right* with $P < .0001$ (*) compared to MeOH solvent control and $P < .0001$ (**) compared to 15 nM HC-toxin. With respect to Fig. 5*B*, photographs presented show NB cells after invasion of the Matrigel and migration through the pores of the membrane (Olympus, 200 x). Nuclei of 10 representative fields per membrane were counted at 200 x magnification. The experiment was repeated independently three times. The mean percentage number of invading cells for each cell line and SDs are presented in the bar graphs shown at *right* with $P = .0002$ (*) compared to MeOH solvent controls.

Example 7

Activation of RB tumor suppression pathways in NB cells.

We next studied the impact of HC-toxin upon proteins regulating G_1/S cell cycle progression. RB tumor suppression activity and regulation of its targets play a major role in G_1-S cell cycle progression and have been found to be deregulated in many human cancers (21). We hypothesized that HC-toxin mediated G_0/G_1 cell cycle arrest is associated with activation of RB tumor suppression pathways. To verify this hypothesis, RB phosphorylation pattern was examined in a time kinetic covering 24 h to 48 h and 120 h of HC-toxin treatment in four asynchronously cycling *MYCN* amplified NB cells. BE(2)-C NB cells treated with 15 or 20 nM HC-toxin displayed a significantly stronger hypophosphorylated RB (pRB) fraction compared to MeOH treated cells (Fig. 6*A*). This effect persisted at all time points investigated. Similar effects were induced by HC-toxin in Kelly, LA-N-1, and NGPNB cells (data not shown).

A strong dose-dependent induction of $p21^{cip1/waf-1}$ at 24 h of HC-toxin treatment was observed in all NB cell lines investigated. After 120 h of HC toxin treatment, $p21^{cip1/waf-1}$ was down-regulated by HC-toxin treatment and therefore appeared to be contra-regulated to $p27^{kip1}$.

RB induced G_1 arrest has been shown to be mediated via repression of E2F target genes necessary for cells to enter S phase (35). Consequently, expression of E2F1 - a member of the E2F transcription factor family but also an E2F target - and of the E2F targets Survivin, Mad2 and N-myc was assessed. Low expression of N-myc and Survivin in solvent treated BE(2)-C NB cells after 24 h of treatment may be ascribable to the early time point in culture. Expression of all listed proteins was significantly down-regulated in HC-toxin treated BE(2)-C cells compared to controls (Fig. 6*A*). . Similar results were obtained in *MYCN* amplified Kelly, LA-N-1 and NGPNB cells (data not shown). These findings demonstrate that HC-toxin

mediated activation of RB function leads to reduced expression of E2F1 and the E2F targets Survivin, Mad2 and N-myc in *MYCN* amplified NB cells.

Apart from regulating the E2F transcription factor family and its targets, RB activity controls the RB-Skp2-$p27^{kip1}$ pathway (36). Therefore, the influence of HC-toxin upon Skp2 and $p27^{kip1}$ protein expression was investigated. A dose-dependent markedly down-regulation of Skp2 oncoprotein consistent with a dose-dependent up-regulation of the cyclin dependent kinase inhibitor $p27^{kip1}$ was measured in HC-toxin treated BE(2)-C NB cells (Fig. 6*A*). Activation of the Skp2-$p27^{kip1}$ by HC-toxin was also observed in *MYCN* amplified Kelly, LA-N-1 and NGP NB cells (data not shown). These findings indicate a HC-toxin mediated activation of the Skp2-$p27^{kip1}$ signalling pathway in NB cells.

The impact of functional $p15^{INK4b}$ and $p16^{INK4a}$ upon RB activity has been demonstrated (25). We hypothesized that induction of these INK4 proteins occurs after longer treatment. Western analysis revealed that both INK4 proteins are significantly up-regulated after 120 h of HC-toxin treatment in a dose-dependent manner in BE(2)-C (Fig. 6*B*) and Kelly and NGP NB cells (data not shown). With respect to Fig. 6*B*, unsynchronized actively growing BE(2)-C cells were treated with indicated concentrations of HC-toxin (HCT) or MeOH solvent control, and protein lysates were harvested at the individual times for Western analysis with ß-actin serving as loading control. The results strengthen the hypothesis that HC-toxin enhances $p15^{INK4b}$/ RB and $p16^{INK4a}$/ RB pathway activity.

Example 8

Tumor cell selective modulation of DNA replication, G_1/S cell cycle progression and RB activity.

We chose primary human skin fibroblasts (PHSFs) to investigate the influence of HC-toxin upon untransformed cells. Western analysis revealed an induction of acetylation of histone 4 after 6 h comparable to that seen in neuroblasts (Fig. 7*A*, *upper panel*). However, the number of viable PHSFs during HC-toxin treatment remained unchanged (Fig. 7*A*). With respect to Fig. 7*A*, harvested cells were evaluated for number and viability by trypan blue exclusion. Results presented are means ± SDs of three independent experiments performed in triplicates. Induction of histone 4 acetylation (panAc-H4) by HC-toxin is shown in the *upper panel*. Cells were treated with various concentrations of HC-toxin (HCT) and MeOH solvent control (% v/v) for 6 h or remained untreated (ut). Proteins were extracted and analyzed for panAc-H4 by Western analysis with Coomassie stain of the gel

serving as loading control.Cytometry analysis including the parameters cell cycle, number of viable and apoptotic cells as well as granularity detected no differences between MeOH solvent and HC-toxin treated PHSFs (Fig. 7*B*). With respect to Fig. 7*B*, cells were treated with different concentrations of HC-toxin or MeOH solvent control for indicated time periods. Changes in phases of the cell cycle and in apoptosis were analyzed as described in Example 1. Shown *at right* are exemplarily FACS profiles of solvent control and HC-toxin treated cells after 72 h of exposure. All parameters investigated remain unaltered after 72 h of treatment. Striped areas represent S phase cells. Accordingly, phosphorylation of RB remained unaltered despite HC-toxin treatment (Fig. 7*C*). With respect to Fig. 7*C*, unsynchronized actively growing PHSFs were treated with 20 nM HC-toxin or MeOH solvent control, and protein lysates were harvested after 24 h of treatment for Western analysis with ß-actin serving as loading control. In conclusion, HC-toxin promotes no detectable effects upon PHSFs.

It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

REFERENCES

1. Cooper, M. J., Hutchins, G. M., Cohen, P. S., Helman, L. J., Mennie, R. J., and Israel, M. A. Human neuroblastoma tumor cell lines correspond to the arrested differentiation of chromaffin adrenal medullary neuroblasts. Cell Growth Differ, *1:* 149-159, 1990.

2. Shimada, H., Ambros, I. M., Dehner, L. P., Hata, J., Joshi, V. V., Roald, B., Stram, D. O., Gerbing, R. B., Lukens, J. N., Matthay, K. K., and Castleberry, R. P. The International Neuroblastoma Pathology Classification (the Shimada system). Cancer, *86:* 364-372, 1999.

3. Escobar, M. A., Grosfeld, J. L., Powell, R. L., West, K. W., Scherer, L. R., 3rd, Fallon, R. J., and Rescorla, F. J. Long-term outcomes in patients with stage IV neuroblastoma. J Pediatr Surg, *41:* 377-381, 2006.

4. Schwab, M., Alitalo, K., Klempnauer, K. H., Varmus, H. E., Bishop, J. M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature, *305:* 245-248, 1983.

5. Fong, C. T., Dracopoli, N. C., White, P. S., Merrill, P. T., Griffith, R. C., Housman, D. E., and Brodeur, G. M. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. Proc Natl Acad Sci U S A, *86:* 3753-3757, 1989.

6. Mertens, F., Johansson, B., Hoglund, M., and Mitelman, F. Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. Cancer Res, *57:* 2765-2780, 1997.

7. Van Roy, N., Laureys, G., Cheng, N. C., Willem, P., Opdenakker, G., Versteeg, R., and Speleman, F. 1;17 translocations and other chromosome 17 rearrangements in human primary neuroblastoma tumors and cell lines. Genes Chromosomes Cancer, *10:* 103-114, 1994.

8. Jenuwein, T. and Allis, C. D. Translating the histone code. Science, *293:* 1074-1080, 2001.

9. Fraga, M. F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K., Iyer, N. G., Perez-Rosado, A., Calvo, E., Lopez, J. A., Cano, A., Calasanz, M. J., Colomer, D., Piris, M. A., Ahn, N., Imhof, A., Caldas, C., Jenuwein, T., and Esteller, M. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet, *37:* 391-400, 2005.

10. Yoo, C. B. and Jones, P. A. Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov, *5:* 37-50, 2006.

11. Rocchi, P., Tonelli, R., Camerin, C., Purgato, S., Fronza, R., Bianucci, F., Guerra, F., Pession, A., and Ferreri, A. M. p21Waf1/Cip1 is a common target induced by short-chain fatty acid HDAC inhibitors (valproic acid, tributyrin and sodium butyrate) in neuroblastoma cells. Oncol Rep, *13:* 1139-1144, 2005.

12. Nebbioso, A., Clarke, N., Voltz, E., Germain, E., Ambrosino, C., Bontempo, P., Alvarez, R., Schiavone, E. M., Ferrara, F., Bresciani, F., Weisz, A., de Lera, A. R., Gronemeyer, H., and Altucci, L. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. Nat Med, *11:* 77-84, 2005.

13. Gottlicher, M., Minucci, S., Zhu, P., Kramer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., and Heinzel, T. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. Embo J, *20:* 6969-6978, 2001.

14. Kelly, W. K., Richon, V. M., O'Connor, O., Curley, T., MacGregor-Curtelli, B., Tong, W., Klang, M., Schwartz, L., Richardson, S., Rosa, E., Drobnjak, M., Cordon-Cordo, C., Chiao, J. H., Rifkind, R., Marks, P. A., and Scher, H. Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. Clin Cancer Res, *9:* 3578-3588, 2003.

15. Cinatl, J., Jr., Cinatl, J., Driever, P. H., Kotchetkov, R., Pouckova, P., Kornhuber, B., and Schwabe, D. Sodium valproate inhibits in vivo growth of human neuroblastoma cells. Anticancer Drugs, *8:* 958-963, 1997.

16. Gore, S. D., Weng, L. J., Figg, W. D., Zhai, S., Donehower, R. C., Dover, G., Grever, M. R., Griffin, C., Grochow, L. B., Hawkins, A., Burks, K., Zabelena,

Y., and Miller, C. B. Impact of prolonged infusions of the putative differentiating agent sodium phenylbutyrate on myelodysplastic syndromes and acute myeloid leukemia. Clin Cancer Res, *8:* 963-970, 2002.

17. Perrine, S. P., Olivieri, N. F., Faller, D. V., Vichinsky, E. P., Dover, G. J., and Ginder, G. D. Butyrate derivatives. New agents for stimulating fetal globin production in the beta-globin disorders. Am J Pediatr Hematol Oncol, *16:* 67-71, 1994.

18. Ryan, Q. C., Headlee, D., Acharya, M., Sparreboom, A., Trepel, J. B., Ye, J., Figg, W. D., Hwang, K., Chung, E. J., Murgo, A., Melillo, G., Elsayed, Y., Monga, M., Kalnitskiy, M., Zwiebel, J., and Sausville, E. A. Phase I and pharmacokinetic study of MS-275, a histone deacetylase inhibitor, in patients with advanced and refractory solid tumors or lymphoma. J Clin Oncol, *23:* 3912-3922, 2005.

19. Jaboin, J., Wild, J., Hamidi, H., Khanna, C., Kim, C. J., Robey, R., Bates, S. E., and Thiele, C. J. MS-27-275, an inhibitor of histone deacetylase, has marked in vitro and in vivo antitumor activity against pediatric solid tumors. Cancer Res, *62:* 6108-6115, 2002.

20. Walton, J. D. HC-toxin. Phytochemistry, 2006.

21. Weinberg, R. A. The retinoblastoma protein and cell cycle control. Cell, *81:* 323-330, 1995.

22. Hernando, E., Nahle, Z., Juan, G., Diaz-Rodriguez, E., Alaminos, M., Hemann, M., Michel, L., Mittal, V., Gerald, W., Benezra, R., Lowe, S. W., and Cordon-Cardo, C. Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature, *430:* 797-802, 2004.

23. Lutz, W., Stohr, M., Schurmann, J., Wenzel, A., Lohr, A., and Schwab, M. Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene, *13:* 803-812, 1996.

24. de Veas, R. G., Schweigerer, L., and Medina, M. A. Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression in

paediatric tumour cells. Effects of tumour cell proliferation modulators on gelatinolytic activity. J Cancer Res Clin Oncol, *121:* 275-278, 1995.

25. Jiang, H., Chou, H. S., and Zhu, L. Requirement of cyclin E-Cdk2 inhibition in p16(INK4a)-mediated growth suppression. Mol Cell Biol, *18:* 5284-5290, 1998.

26. Ehemann, V., Hashemi, B., Lange, A., and Otto, H. F. Flow cytometric DNA analysis and chromosomal aberrations in malignant glioblastomas. Cancer Lett, *138:* 101-106, 1999.

27. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods, *139:* 271-279, 1991.

28. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res, *47:* 3239-3245, 1987.

29. Sadee, W., Yu, V. C., Richards, M. L., Preis, P. N., Schwab, M. R., Brodsky, F. M., and Biedler, J. L. Expression of neurotransmitter receptors and myc protooncogenes in subclones of a human neuroblastoma cell line. Cancer Res, *47:* 5207-5212, 1987.

30. Hockfield, S. and McKay, R. D. Identification of major cell classes in the developing mammalian nervous system. J Neurosci, *5:* 3310-3328, 1985.

31. Dahlstrand, J., Collins, V. P., and Lendahl, U. Expression of the class VI intermediate filament nestin in human central nervous system tumors. Cancer Res, *52:* 5334-5341, 1992.

32. Rutka, J. T., Ivanchuk, S., Mondal, S., Taylor, M., Sakai, K., Dirks, P., Jun, P., Jung, S., Becker, L. E., and Ackerley, C. Co-expression of nestin and vimentin intermediate filaments in invasive human astrocytoma cells. Int J Dev Neurosci, *17:* 503-515, 1999.

33. Thomas, S. K., Messam, C. A., Spengler, B. A., Biedler, J. L., and Ross, R. A. Nestin is a potential mediator of malignancy in human neuroblastoma cells. J Biol Chem, *279:* 27994-27999, 2004.

34. Walton, J. D., Kattan, D. R., Thomas, S. K., Spengler, B. A., Guo, H. F., Biedler, J. L., Cheung, N. K., and Ross, R. A. Characteristics of stem cells from human neuroblastoma cell lines and in tumors. Neoplasia, *6:* 838-845, 2004.

35. Sherr, C. J. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res, *60:* 3689-3695, 2000.

36. Ji, P., Jiang, H., Rekhtman, K., Bloom, J., Ichetovkin, M., Pagano, M., and Zhu, L. An Rb-Skp2-p27 pathway mediates acute cell cycle inhibition by Rb and is retained in a partial-penetrance Rb mutant. Mol Cell, *16:* 47-58, 2004.

37. Ferguson, K. L., Vanderluit, J. L., Hebert, J. M., McIntosh, W. C., Tibbo, E., MacLaurin, J. G., Park, D. S., Wallace, V. A., Vooijs, M., McConnell, S. K., and Slack, R. S. Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. Embo J, *21:* 3337-3346, 2002.

38. Slack, R. S., El-Bizri, H., Wong, J., Belliveau, D. J., and Miller, F. D. A critical temporal requirement for the retinoblastoma protein family during neuronal determination. J Cell Biol, *140:* 1497-1509, 1998.

39. Lee, E. Y., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature, *359:* 288-294, 1992.

40. Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M., van der Valk, M., Hooper, M. L., Berns, A., and te Riele, H. Requirement for a functional Rb-1 gene in murine development. Nature, *359:* 328-330, 1992.

41. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. Effects of an Rb mutation in the mouse. Nature, *359:* 295-300, 1992.

42. Morris, E. J. and Dyson, N. J. Retinoblastoma protein partners. Adv Cancer Res, *82:* 1-54, 2001.

43. Ferguson, K. L. and Slack, R. S. The Rb pathway in neurogenesis. Neuroreport, *12:* A55-62, 2001.

44. Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature, *391:* 597-601, 1998.

45. Luo, R. X., Postigo, A. A., and Dean, D. C. Rb interacts with histone deacetylase to repress transcription. Cell, *92:* 463-473, 1998.

46. Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature, *391:* 601-605, 1998.

47. Siddiqui, H., Solomon, D. A., Gunawardena, R. W., Wang, Y., and Knudsen, E. S. Histone deacetylation of RB-responsive promoters: requisite for specific gene repression but dispensable for cell cycle inhibition. Mol Cell Biol, *23:* 7719-7731, 2003.

48. Minucci, S. and Pelicci, P. G. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer, *6:* 38-51, 2006.

49. Nguyen, D. X., Baglia, L. A., Huang, S. M., Baker, C. M., and McCance, D. J. Acetylation regulates the differentiation-specific functions of the retinoblastoma protein. Embo J, *23:* 1609-1618, 2004.

50. Chan, H. M., Krstic-Demonacos, M., Smith, L., Demonacos, C., and La Thangue, N. B. Acetylation control of the retinoblastoma tumour-suppressor protein. Nat Cell Biol, *3:* 667-674, 2001.

51. Ouwehand, K., de Ruijter, A. J., van Bree, C., Caron, H. N., and van Kuilenburg, A. B. Histone deacetylase inhibitor BL1521 induces a G1-phase arrest

in neuroblastoma cells through altered expression of cell cycle proteins. FEBS Lett, *579:* 1523-1528, 2005.

52. Mitsiades, C. S., Poulaki, V., McMullan, C., Negri, J., Fanourakis, G., Goudopoulou, A., Richon, V. M., Marks, P. A., and Mitsiades, N. Novel histone deacetylase inhibitors in the treatment of thyroid cancer. Clin Cancer Res, *11:* 3958-3965, 2005.

53. Sambucetti, L. C., Fischer, D. D., Zabludoff, S., Kwon, P. O., Chamberlin, H., Trogani, N., Xu, H., and Cohen, D. Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. J Biol Chem, *274:* 34940-34947, 1999.

54. Sakimura, R., Tanaka, K., Nakatani, F., Matsunobu, T., Li, X., Hanada, M., Okada, T., Nakamura, T., Matsumoto, Y., and Iwamoto, Y. Antitumor effects of histone deacetylase inhibitor on Ewing's family tumors. Int J Cancer, *116:* 784-792, 2005.

55. Ji P, Jiang H, Rekhtman K, Bloom J, Ichetovkin M, Pagano M, Zhu L. An Rb-Skp2-p27 pathway mediates acute cell cycle inhibition by Rb and is retained in a partial penetrance Rb mutant. Mol Cell 2004; 16(1):47-58.

WHAT IS CLAIMED IS:

1. A method of treating a subject having a tumor, comprising administering a dose of a histone deacetylase (HDAC) inhibitor to a subject, wherein the HDAC inhibitor activates retinoblastoma (RB) tumor suppression networks.

2. The method of claim 1, wherein the tumor suppression pathway is the retinoblastoma (RB) tumor suppression pathway.

3. The method of claim 1, wherein the HDAC inhibitor is selected from a group consisting of carboxylates, hydroxamic acid derivatives, benzamides and tetracyclic peptides.

4. The method of claim 1, wherein the HDAC inhibitor is *Helminthosporium Carbonum* toxin.

5. The method of claim 1, wherein the tumor is a Small Round Blue Cell Tumor.

6. The method of claim 5, wherein the Small Round Blue Cell Tumor is of a group consisting of: Desmoplastic small round blue cell tumor, Ewing's Sarcoma, Acute Leukemia, Small Cell Lung Carcinoma, Small Cell Mesothelioma, Neuroblastoma, Primitive Neuroectodermal tumor, Rhabdomyosarcoma, Wilm's Tumor, and Melanoma.

7. The method of claim 6, wherein the Small Round Blue Cell Tumor is Neuroblastoma.

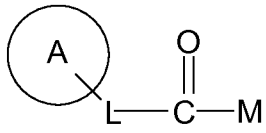
8. The method of claim 1, wherein the subject is a mammal.

9. The method of claim 8, wherein the mammal is of a group consisting of: human, rat, dog, cat, cattle, mouse, guinea pig, gerbil, pig, and sheep.

10. The method of claim 1, wherein the subject is a human.

11. The method of claim 1, wherein the dose of HDAC inhibitor is between 10-20 nM.

12. Use of a compound according to the general formula (I)



(I)

wherein the symbols have the following meanings:

A is a 9- to 15-membered heterocycle optionally carrying one or more double bonds, and comprising 3 to 5 units of the type -D-E-G- wherein

D is selected from -N(R^1)-, -O-, and –S(O)(R^2R^3)$_m$-; and/or

E is selected from methylene -CH_2-, ethylene -CH_2CH_2- and propylene -CH_2-CH_2-CH_2-, wherein in the methylene, ethylene and propylene groups one or more H atoms can be replaced by: linear and branched C_1- to C_{10} alkyl groups, which are optionally substituted by one or more substituents from the group -OH, F, -SH, -SCH_3, -NH_2, -NHC(NH)NH_2, -C(O)OH, -C(O)NH_2, C_6H_5, C_6H_4OH, indolyl, imidazolyl or a salt thereof; and linear and branched C_2- to C_{10} alkylene groups, which are optionally substituted by one or more OH and/or one or more F; or, in case 2 or more even-numbered substituents are present, the named alkyl or alkylene group forms together with a further alkyl or alkylene group on the same or a different carbon atom a 5- to 7-membered cycle; or the named alkyl and alkylene groups form together with the group R^1 a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

G is a keto group -C(O)- or a thioketo group -C(S)-; and/or

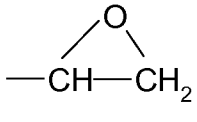
wherein the units -D-E-G- not attached to L can be fully or partially identical with or entirely different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

L is attached to D or E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group in which one or more carbon atoms may be replaced by non adjacent O

atoms, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



hydroxyamino groups –N(OH)H, hydroxymethyl groups -CH_2OH, 1-fluoroethyl groups -$CHFCH_3$ and 1-chloroethyl groups -$CHClCH_3$; and/or

R^1 is selected from: H; linear and branched C_1- to C_{10} alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear or branched C_2- to C_{10} alkylene groups which are optionally substituted by one or more OH and/or one or more F; or R^1 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

R^2 and R^3 are independently of each other selected from: H; linear and branched C_1- to C_{10} alkyl groups which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_{10} alkylene groups, which are optionally substituted by one or more OH and/or one or more F; or R^2 and R^3, together with the sulfur atom to which they are attached, form a 5- to 7-membered cycle; or one of R^2, R^3 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle, which includes the C and the S atom to which the named groups are attached; and/or

m is 0 or 1,

and/ or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for the treatment of Small Round Blue Cell Tumor.

13. The use according to claim 12, wherein the cycle A comprises 3, 4 or 5 amino acids in the D- or L-form

14. The use according to claim 12 or 13, wherein the substituents have the following meanings:

A is a 11- to 13-membered cycle and comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein one or both H atoms can be replaced by: a linear or branched C_1- to C_4 alkyl group, which are optionally substituted by one or more substituents from the group -OH, F, -SH, $-SCH_3$, $-NH_2$, $-NHC(NH)NH_2$, $-C(O)OH$, $-C(O)NH_2$, C_6H_5, C_6H_4OH, indolyl, imidazolyl or a salt thereof; and/or a linear or branched C_2- to C_4 alkylene group, which is optionally substituted by one or more OH and/or one or more F; or the named alkyl and alkylene groups form together with the group R^1 a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached; and/or

G is a keto group -C(O)-; and/or

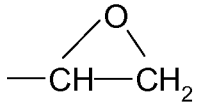
wherein the units -D-E-G- not attached to L can be fully or partially identical with or entirely different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is selected from: H; linear and branched C_1- to C_4 alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_4 alkylene groups which are optionally substituted by one or more OH and/or one or more F; or R^1 forms together with one alkyl or alkylene group in E a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached, and/or

L is attached to E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group in which one or more carbon atoms may be replaced by non adjacent O atoms, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



and hydroxyamino groups –N(OH)H.

15. The use according to claim 13, wherein the cycle A comprises 4 natural amino acids in the L- or D-form.

16. The use according to claim 13 or 14, wherein the substituents have the following meanings:

A is a 12-membered cycle and comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein in one or two units -D-E-G-, one or both H atoms can be replaced by: a linear or branched C_1- to C_4 alkyl group and/or a linear or branched C_2- to C_4 alkylene group; and wherein in only one unit -D-E-G-, one H atom in $-CH_2-$ is replaced by a linear or branched C_1- to C_4 alkyl group or a linear or branched C_2- to C_4 alkylene group, with the said alkyl or alkylene group forming together with R^1 in the neighbouring NR^1 group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

G is a keto group -C(O)-; and/or

at least 2 units -D-E-G- are different from each other; and/or

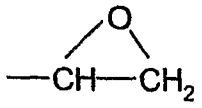
the cycle A can comprise one or more double bonds; and/or

R^1 is selected from: H; linear and branched C_1- to C_4 alkyl groups, which are optionally substituted by one or more OH and/or one or more F; and linear and branched C_2- to C_4 alkylene groups, which are optionally substituted by one or more OH and/or one or more F; and wherein in exactly one unit -D-E-G-, R^1 forms together with the said alkyl or alkylene in the neighbouring $-CH_2-$ group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

L is attached to E in one unit -D-E-G- and is a linear C_3- to C_5-alkyl group, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups

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and hydroxyamino groups –N(OH)H.

17. The use according to claim 16, wherein the substituents have the following meanings:

A is a 12-membered cycle and comprises 4 units of the type -D-E-G- , wherein

D is NR^1; and/or

E is $-CH_2-$, wherein in one or two units -D-E-G-, one or both H atoms can be replaced by: a methyl or ethyl group; and wherein in only one unit -D-E-G-, one H atom in $-CH_2-$ is replaced by a linear or branched C_1- to C_4 alkyl group or a linear or branched C_2- to C_4 alkylene group, and wherein the said alkyl or alkylene group forms together with R^1 in the neighbouring NR^1 group a 5- to 7-membered cycle which includes the C and the N atom to which the named groups are attached;

G is a keto group -C(O)-; and/or

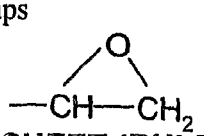
wherein at least 2 units -D-E-G- are different from each other; and/or

the cycle A can comprise one or more double bonds; and/or

R^1 is H or a linear or branched C_1- to C_4-alkyl group in three of the units -D-E-G-; and wherein in the other unit –D-E-G, R^1 forms together with the said alkyl or alkylene in the neighbouring $-CH_2-$ group a 5- to 7-membered cycle, which includes the C and the N atom to which the named groups are attached; and/or

L is attached to E in one unit -D-E-G- and is a C_3- to C_5-alkyl group, and wherein one or more H atom in the alkyl chain may be substituted by one or more alkyl groups; and/or

M is selected from epoxy groups



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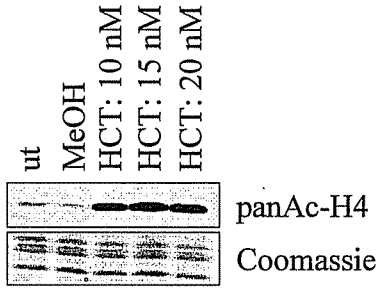
and hydroxyamino groups – N(OH)H.

18. The use of any one of claims 12 to 17, wherein the Small Round Blue Cell Tumor is of a group consisting of: Desmoplastic small round blue cell tumor, Ewing's Sarcoma, Acute Leukemia, Small Cell Lung Carcinoma, Small Cell Mesothelioma, Neuroblastoma, Primitive Neuroectodermal tumor, Rhabdomyosarcoma, Wilm's Tumor, and Melanoma.

RECTIFIED SHEET (RULE 91)
ISA/EP

Figure 1

A


ut
MeOH
HCT: 10 nM
HCT: 15 nM
HCT: 20 nM
panAc-H4
Coomassie

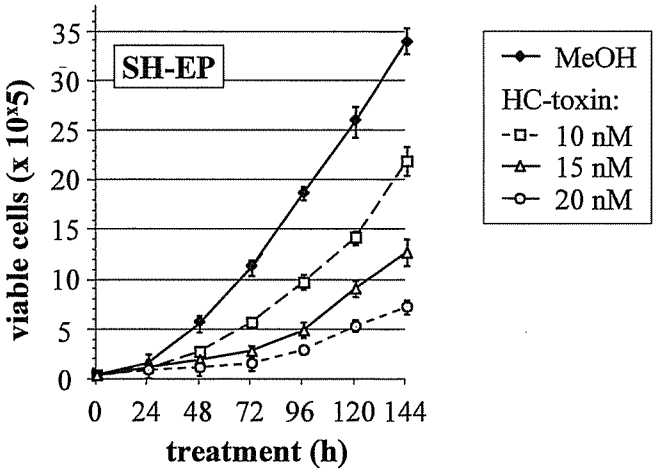

SH-EP
viable cells (x 10^5)
0
5
10
15
20
25
30
35
0 24 48 72 96 120 144
treatment (h)
MeOH
HC-toxin:
10 nM
15 nM
20 nM

Figure 1 *continued*

B

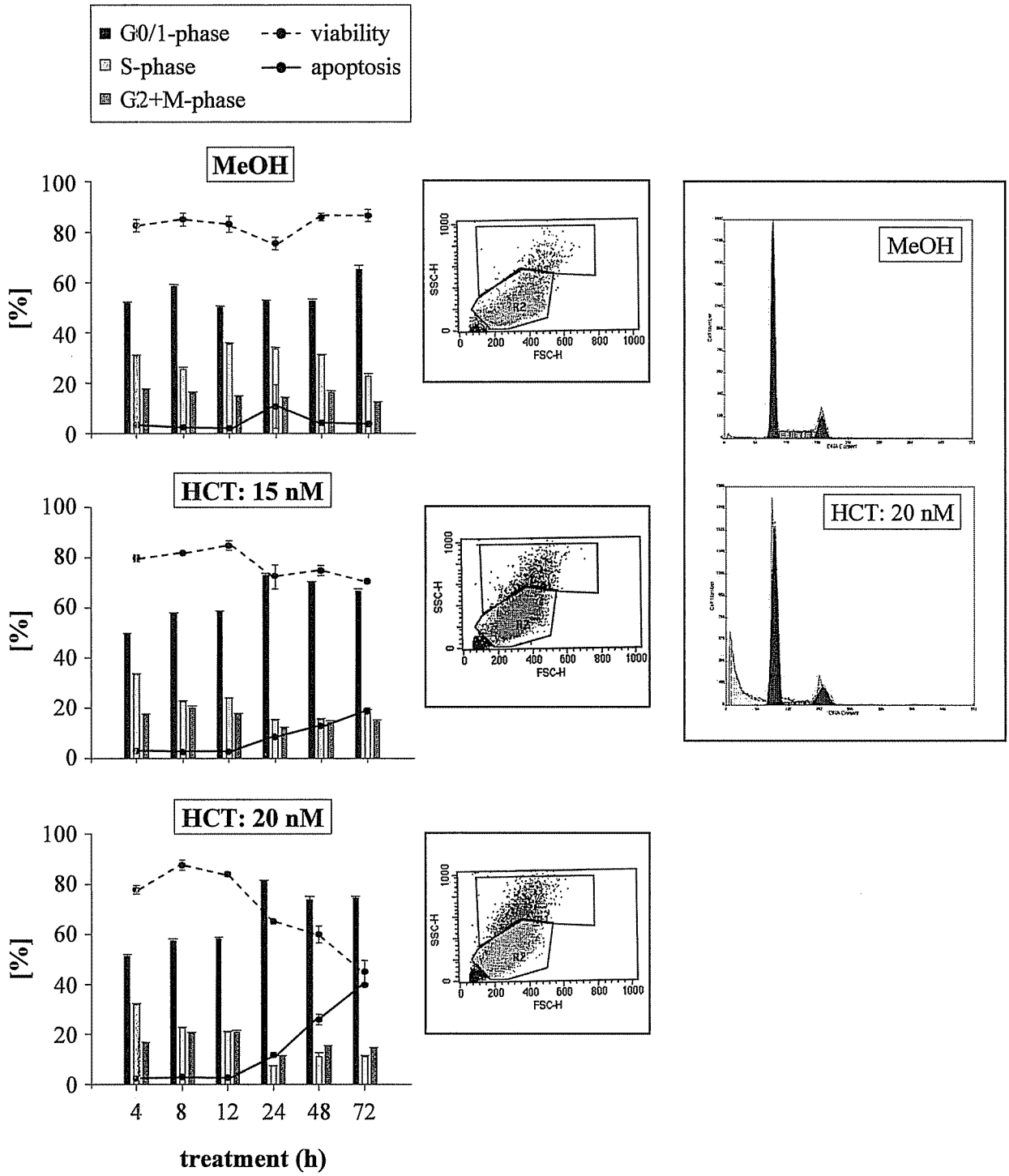


Figure 1 *continued*

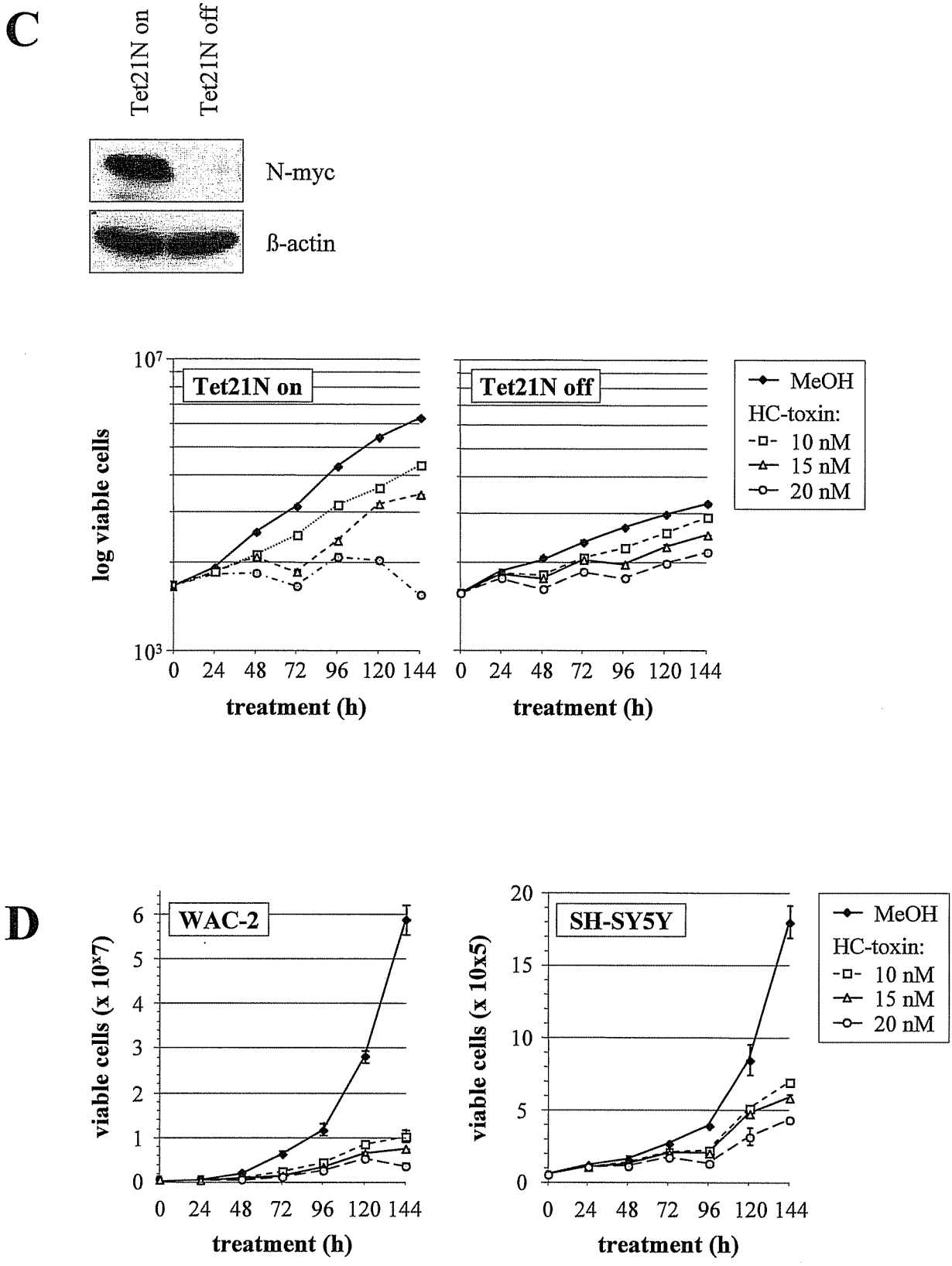
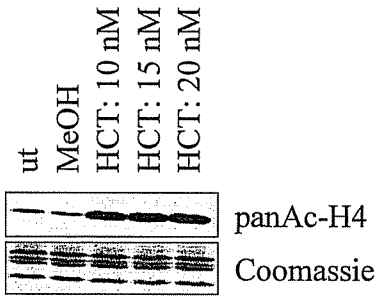


Figure 2

A


ut
MeOH
HCT: 10 nM
HCT: 15 nM
HCT: 20 nM
panAc-H4
Coomassie

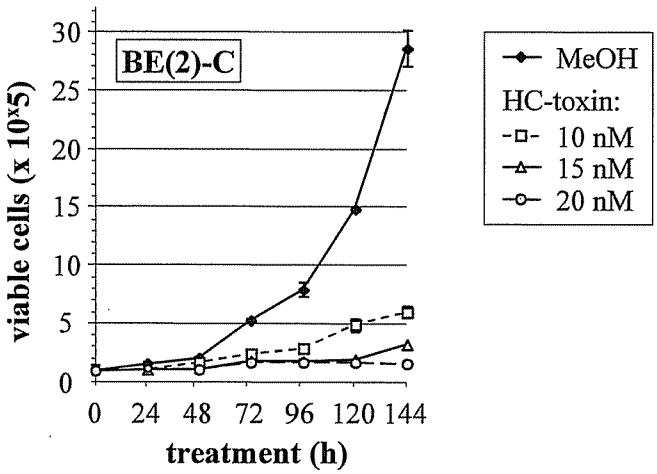

BE(2)-C
viable cells (x 10^5)
30
25
20
15
10
5
0
0 24 48 72 96 120 144
treatment (h)
MeOH
HC-toxin:
10 nM
15 nM
20 nM

Figure 2 *continued*

B

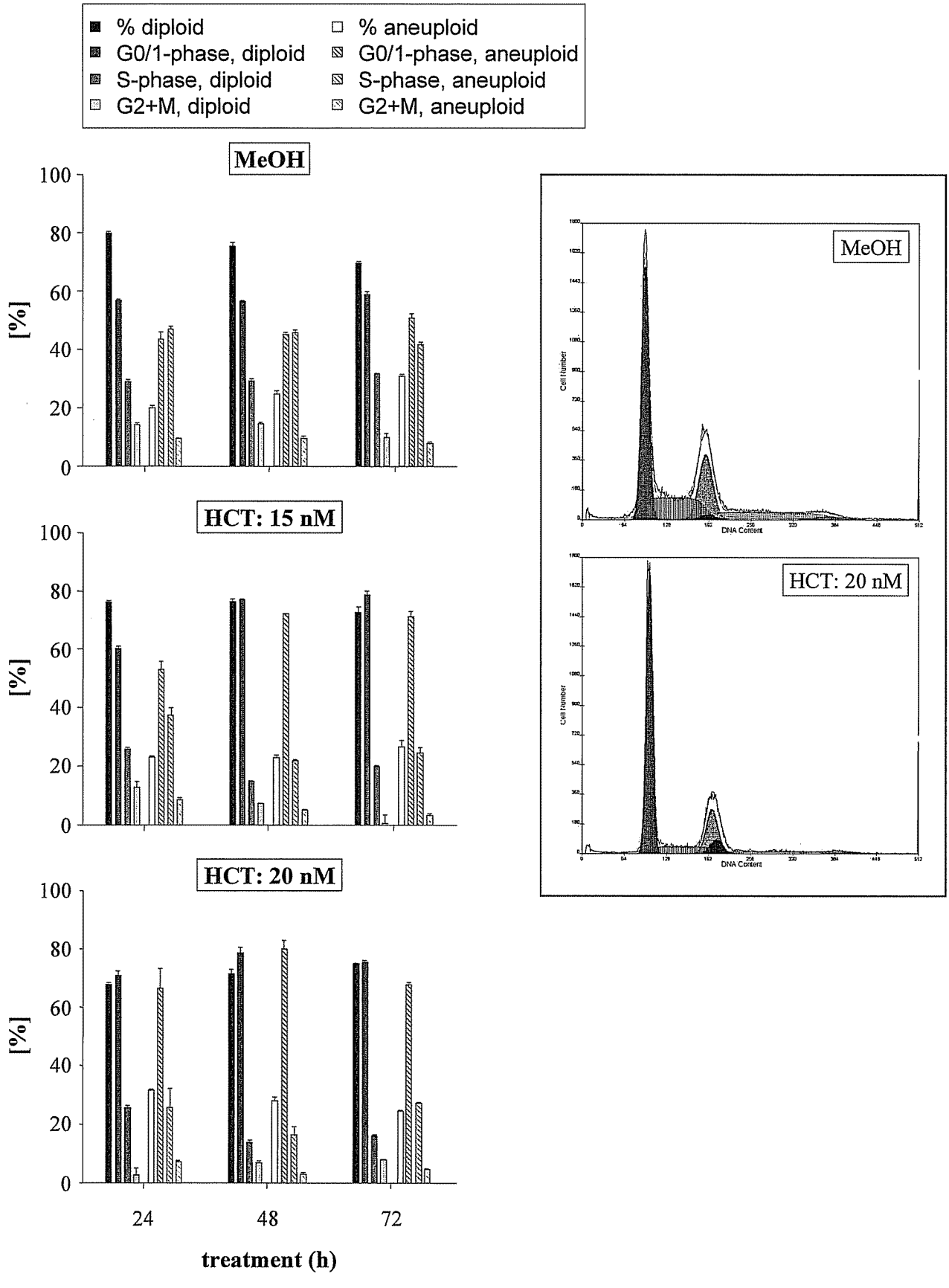
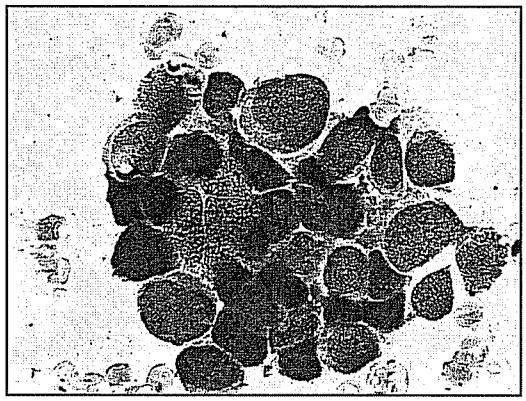


Figure 3

A



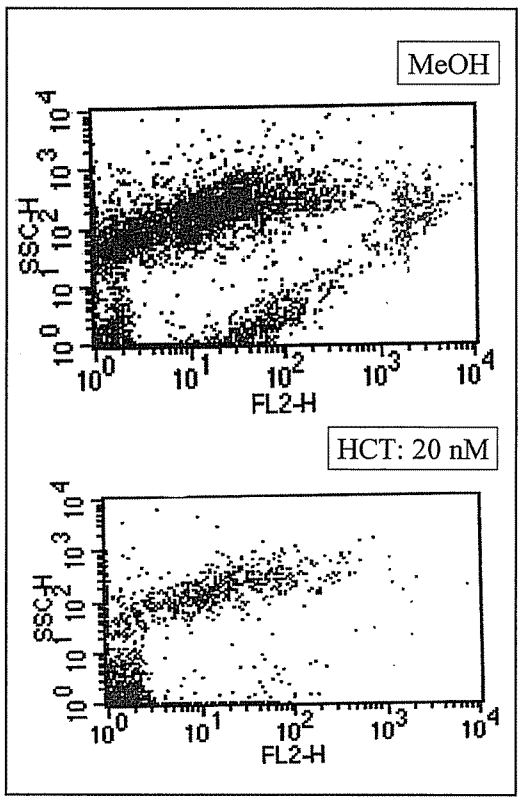


Figure 3 ***continued***

B

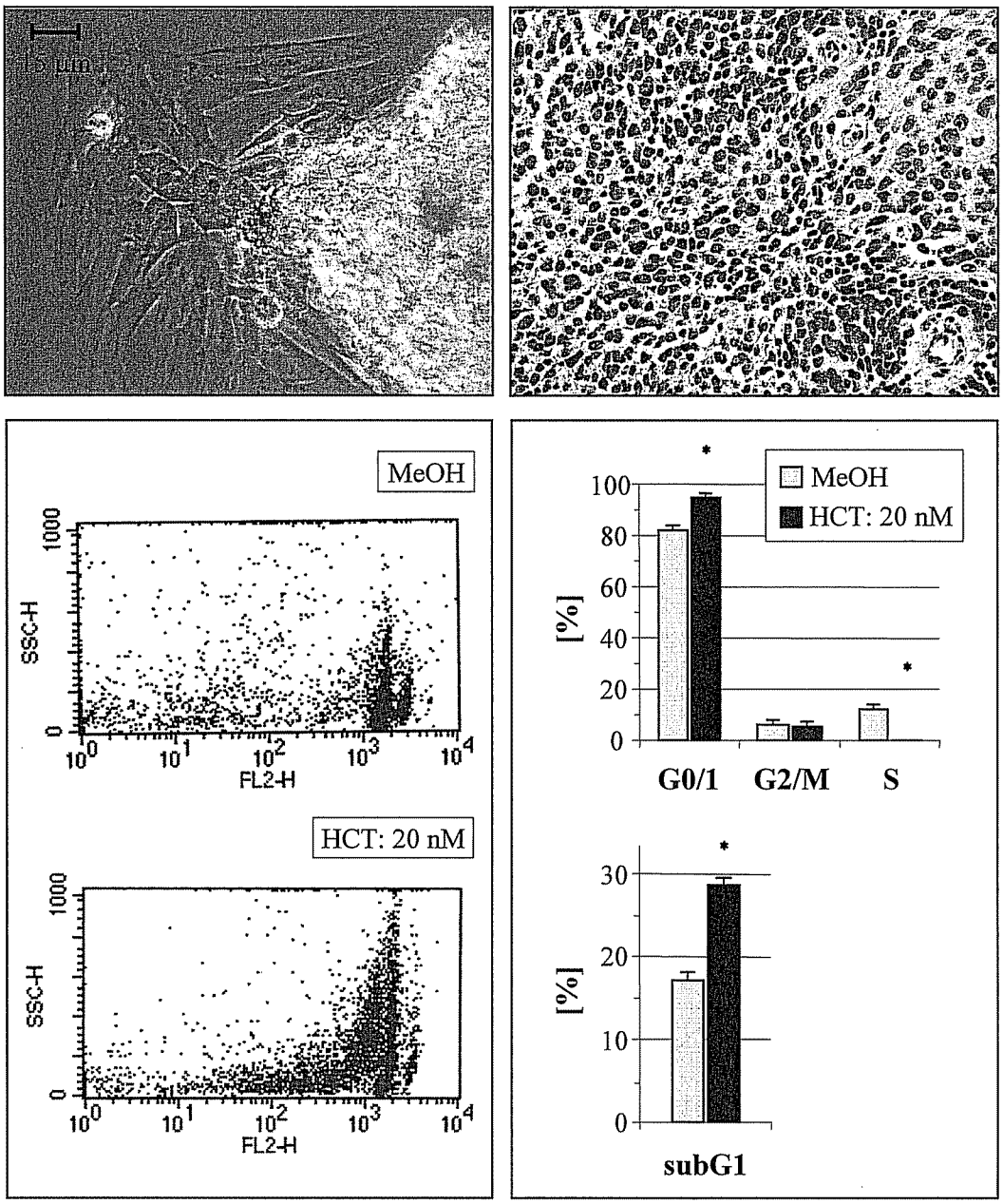


Figure 4

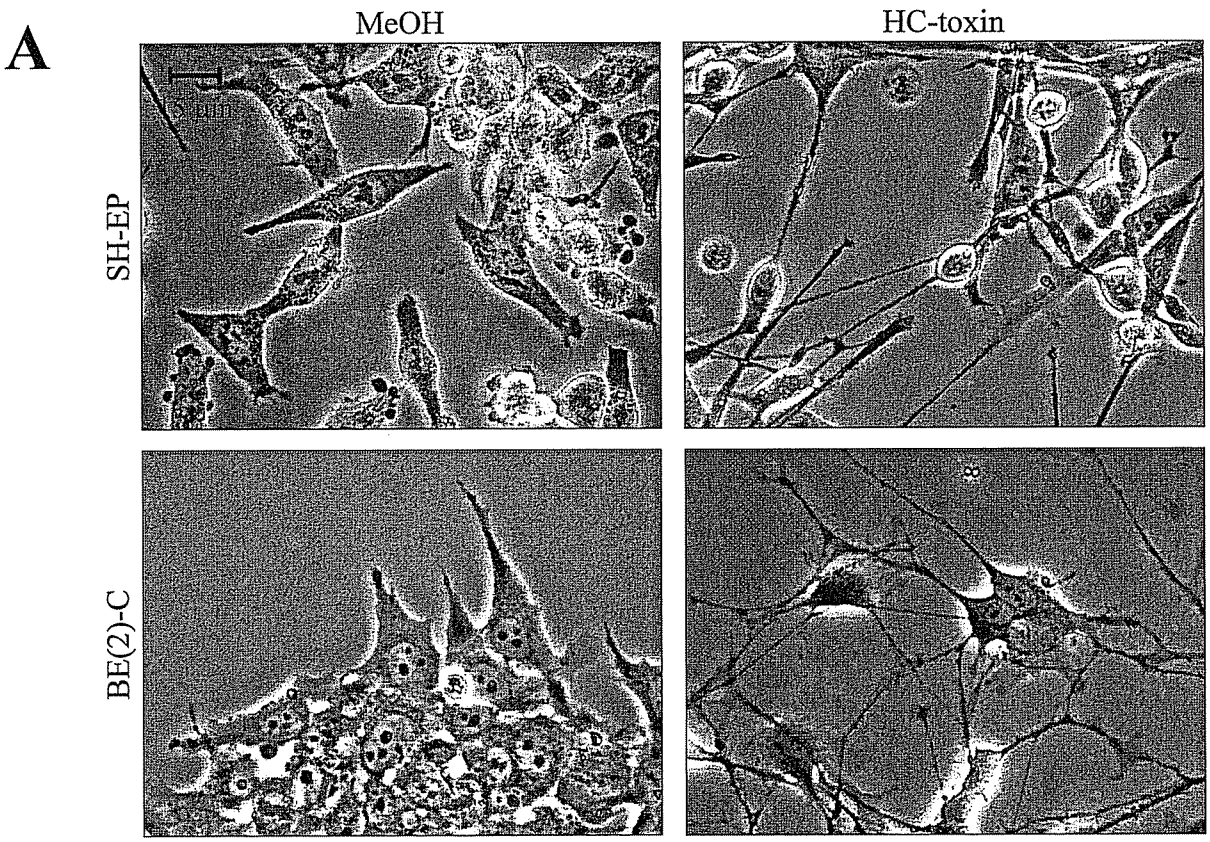
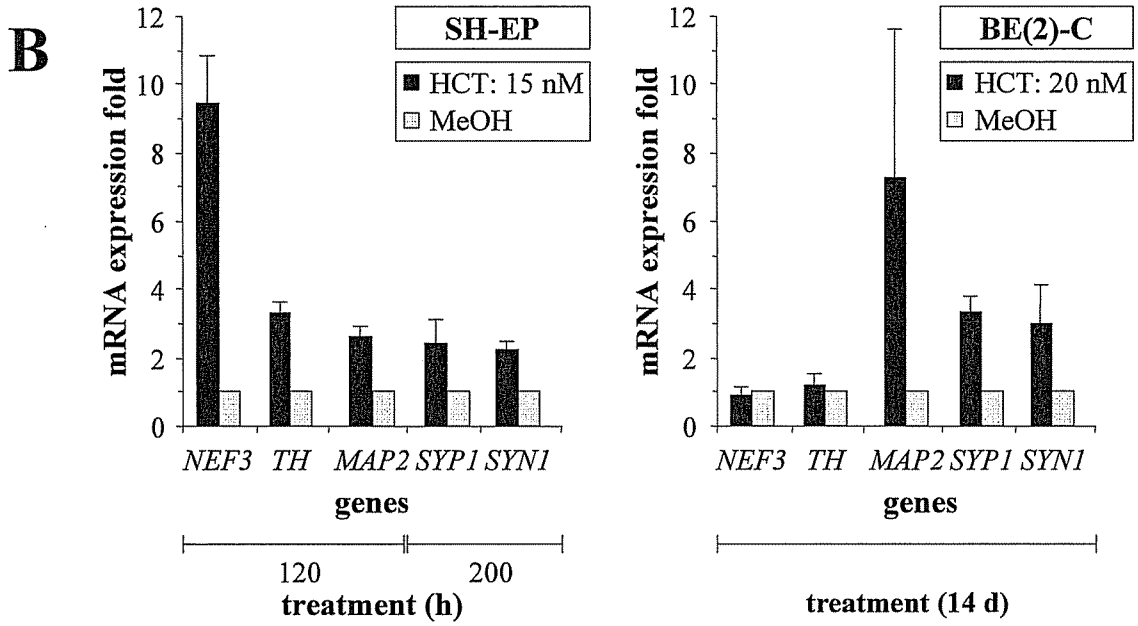


Figure 4 *continued*



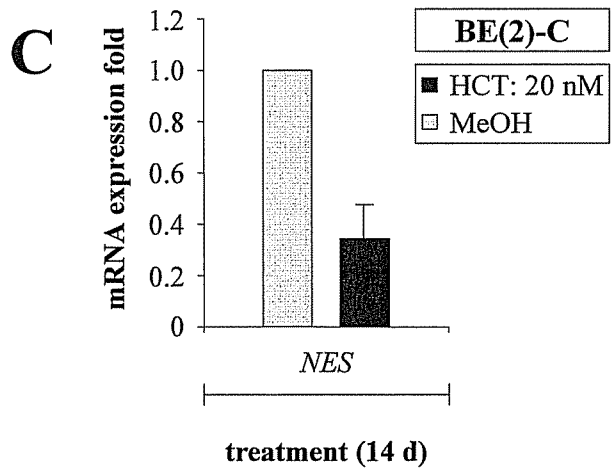


Figure 4 *continued*

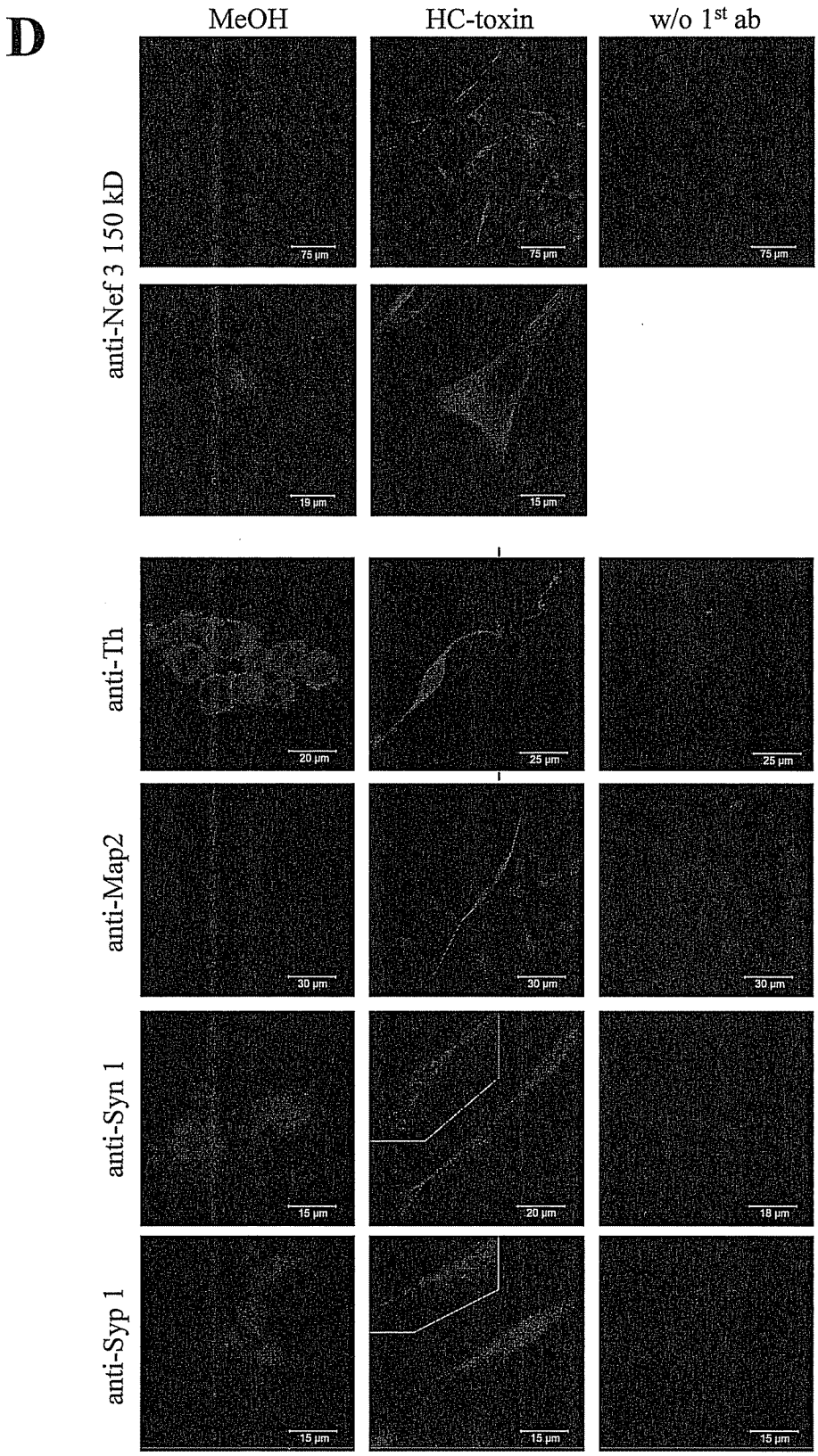


Figure 5

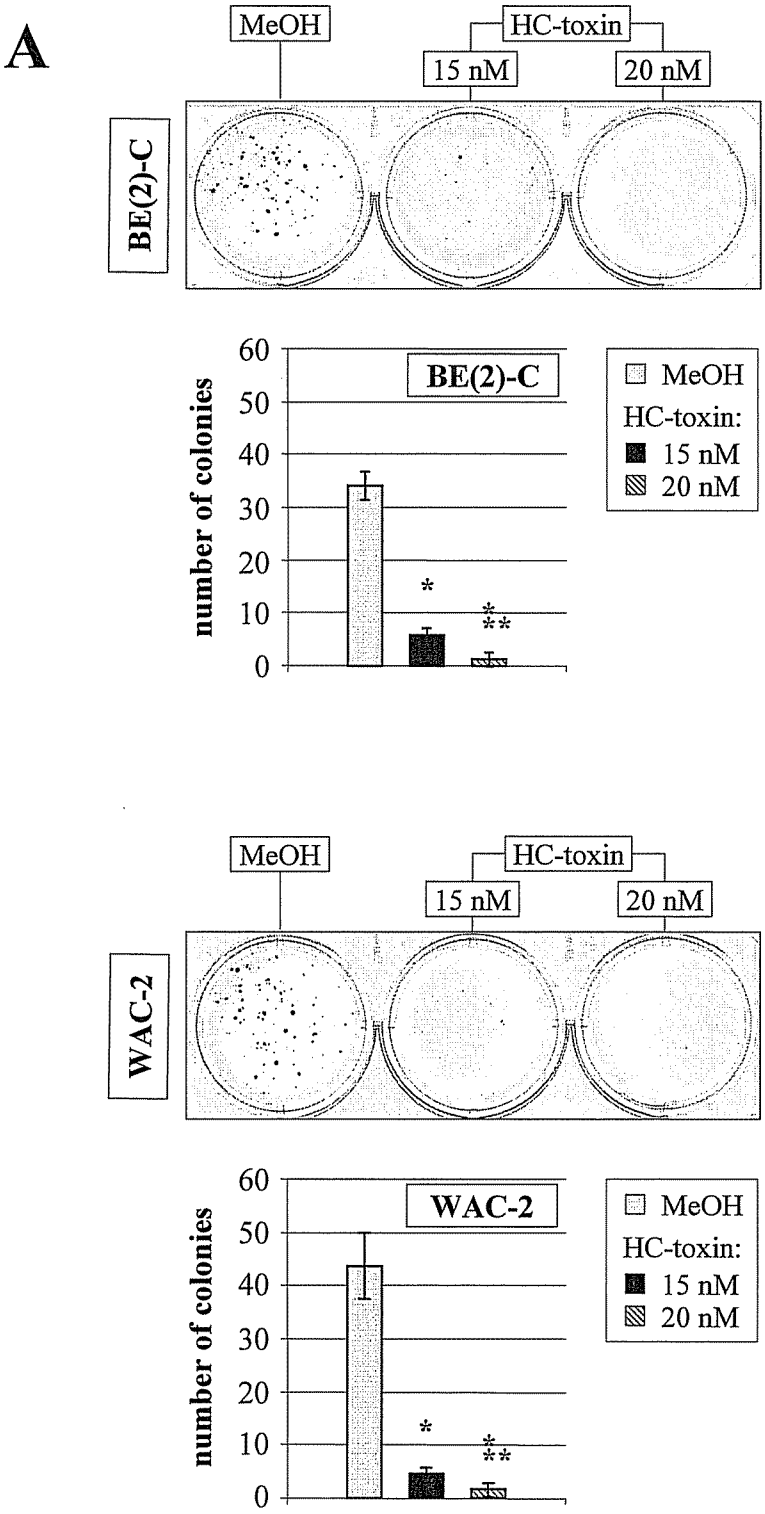
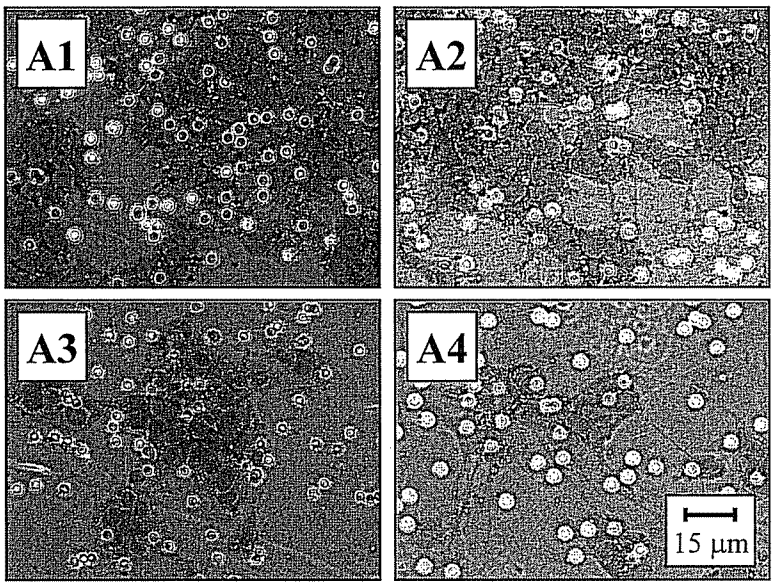
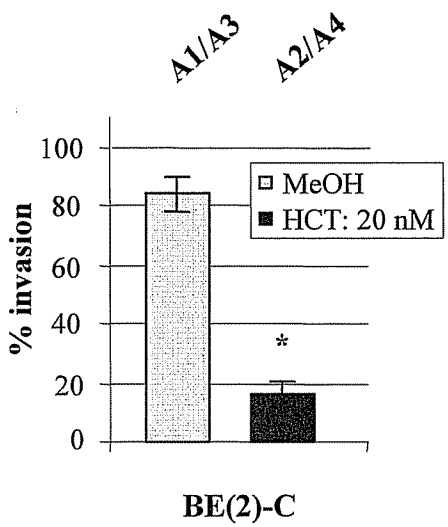
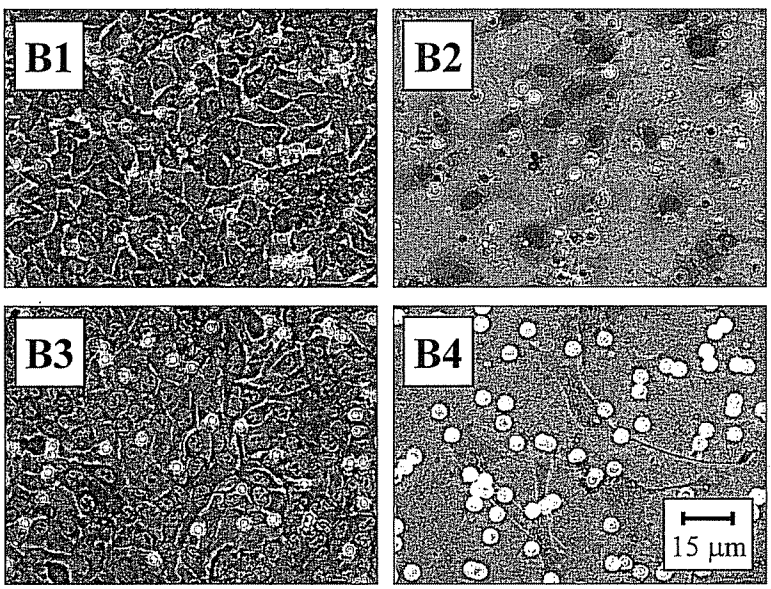

A
MeOH
HC-toxin
15 nM
20 nM
BE(2)-C
number of colonies
60
50
40
30
20
10
0
BE(2)-C
MeOH
HC-toxin:
15 nM
20 nM
*
*
**
MeOH
HC-toxin
15 nM
20 nM
WAC-2
number of colonies
60
50
40
30
20
10
0
WAC-2
MeOH
HC-toxin:
15 nM
20 nM
*
*
**

Figure 5 ***continued***

B







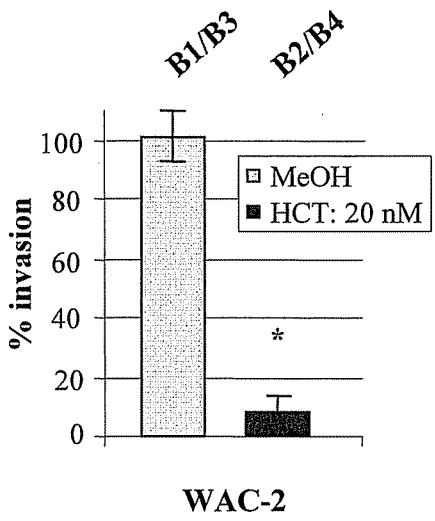


Figure 6

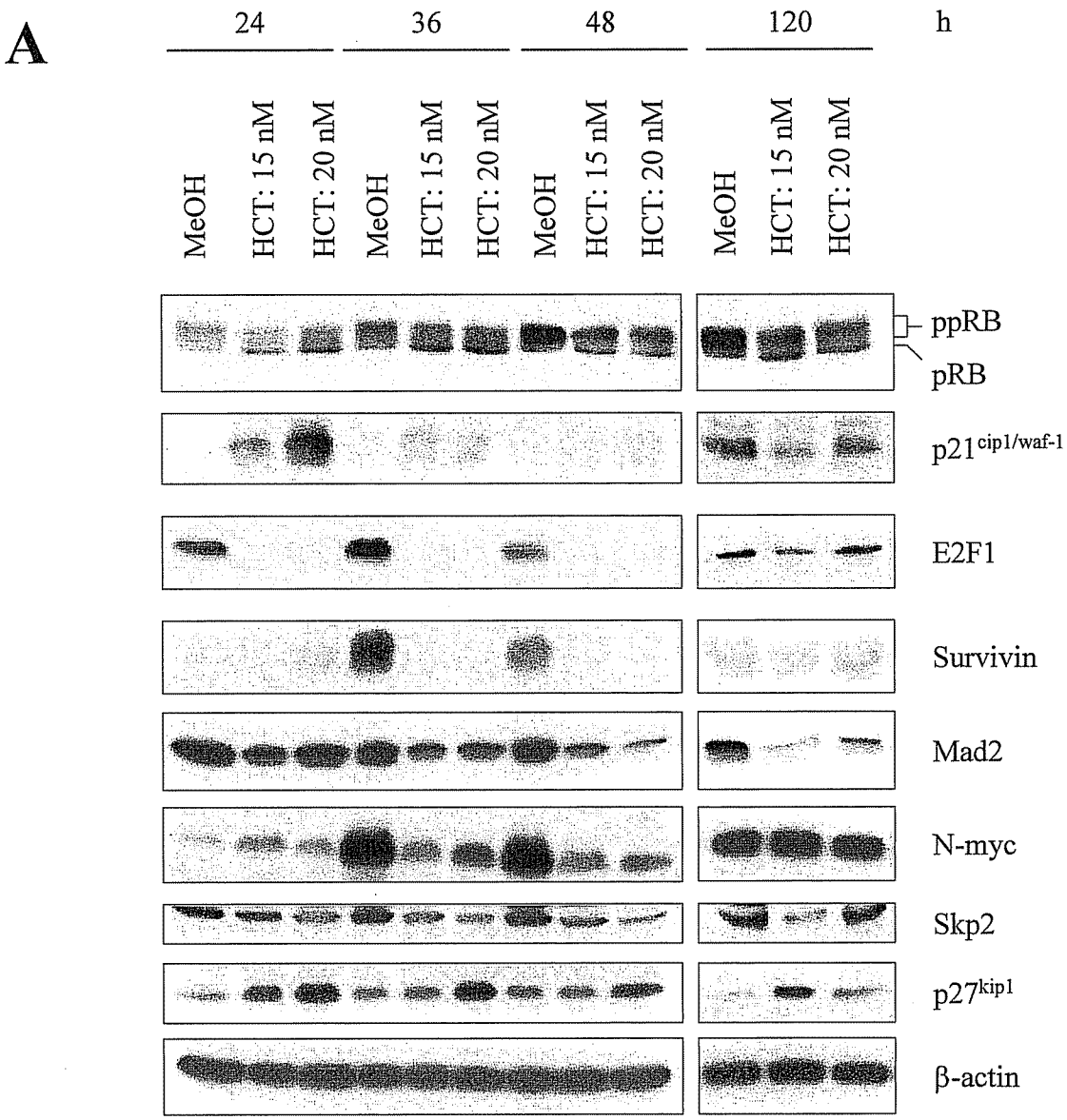

A
24
36
48
120
h
MeOH
HCT: 15 nM
HCT: 20 nM
ppRB
pRB
p21cip1/waf-1
E2F1
Survivin
Mad2
N-myc
Skp2
p27kip1
β-actin

Figure 6 ***continued***

B

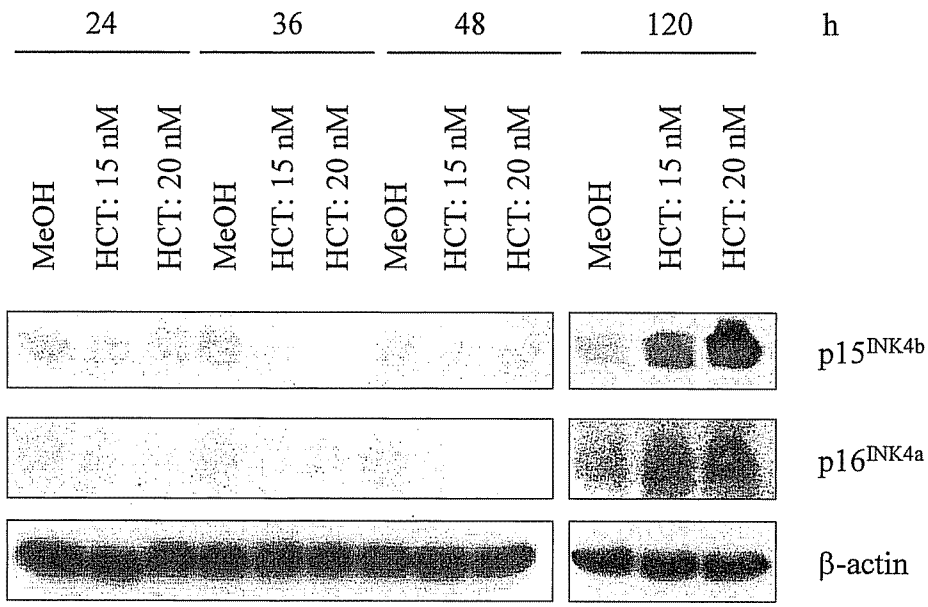
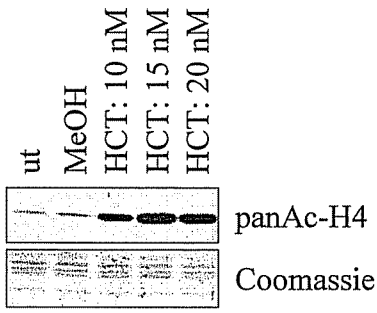


Figure 7

A


ut
MeOH
HCT: 10 nM
HCT: 15 nM
HCT: 20 nM
panAc-H4
Coomassie

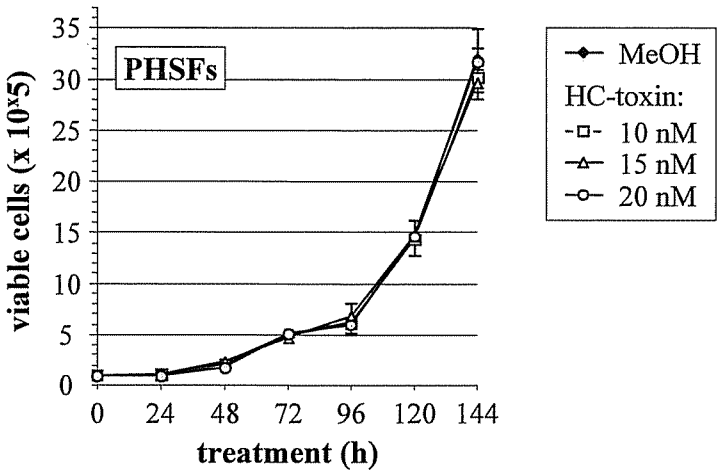

PHSFs
viable cells (x 10^5)
35
30
25
20
15
10
5
0
0
24
48
72
96
120
144
treatment (h)
MeOH
HC-toxin:
10 nM
15 nM
20 nM

Figure 7 *continued*

B

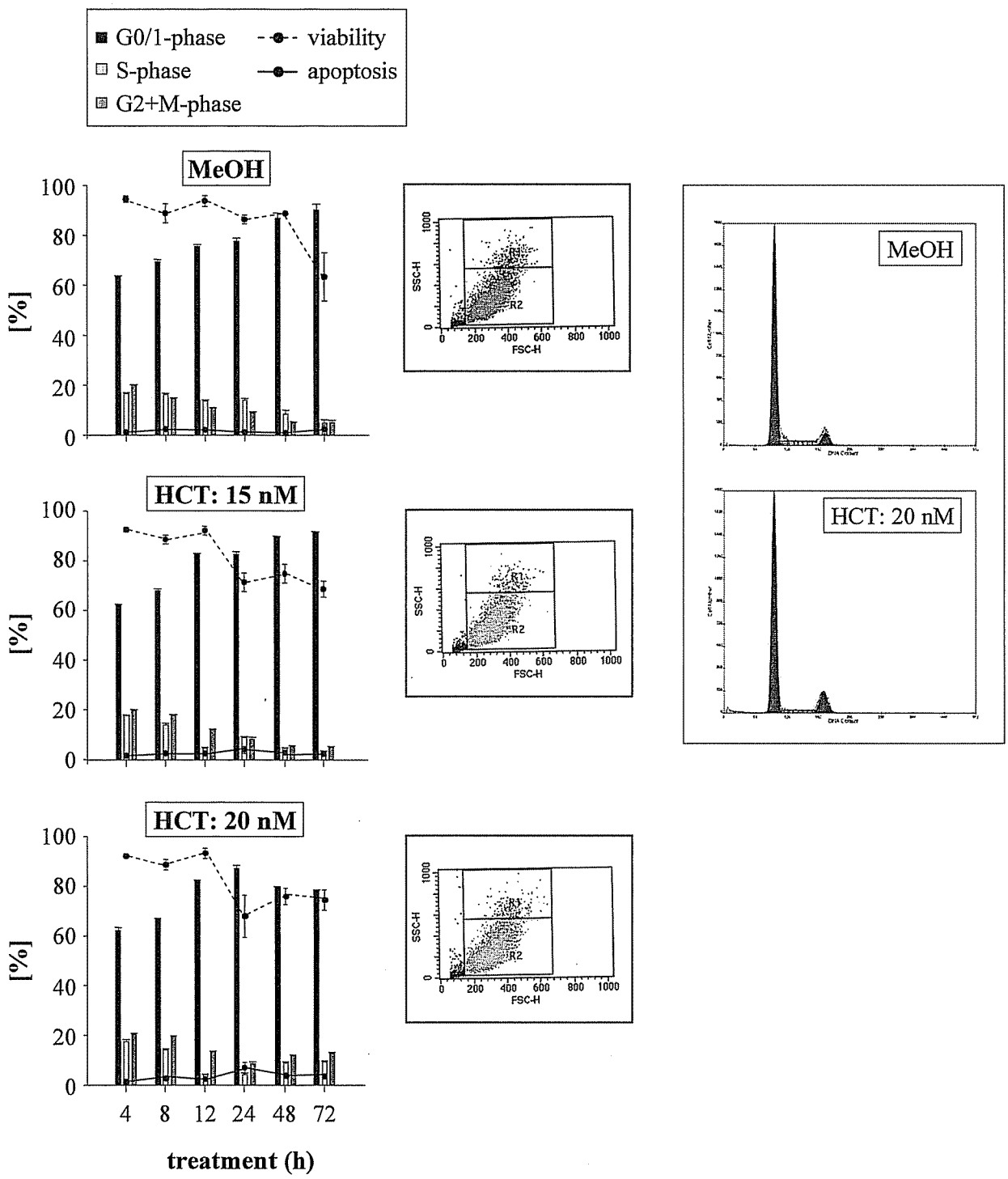
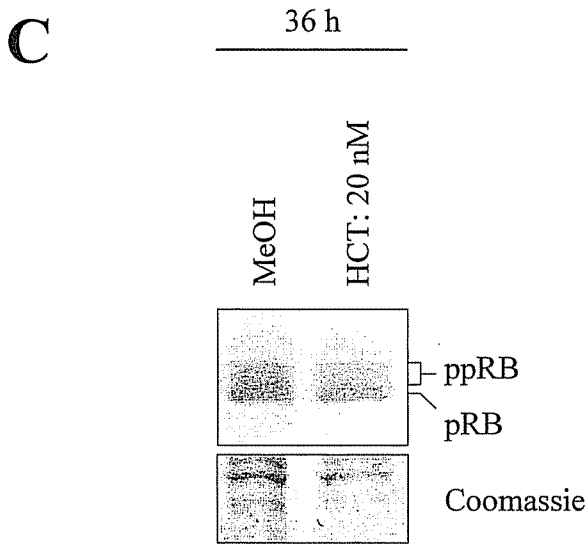


Figure 7 *continued*



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/061206

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/12 C07K5/12 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/117930 A (SLOAN KETTERING INST CANCER [US]; MARKS PAUL A [US]; UNGERSTEDT JOHANN) 15 December 2005 (2005-12-15) claims 1,4,7-9	1-18
X	US 2006/229236 A1 (SATOH SHIGEKI [JP] ET AL) 12 October 2006 (2006-10-12) claims 9-11,20-22	1-6,8-18
X	SAKIMURA RIKU ET AL: "Antitumor effects of histone deacetylase inhibitor on Ewing's family tumors" INTERNATIONAL JOURNAL OF CANCER, vol. 116, no. 5, September 2005 (2005-09), pages 784-792, XP002472112 ISSN: 0020-7136 abstract	1-6,8-18

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 March 2008	25/03/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ganschow, Silke

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOYLE GLEN M ET AL: "Histone deacetylase inhibitors and malignant melanoma." PIGMENT CELL RESEARCH / SPONSORED BY THE EUROPEAN SOCIETY FOR PIGMENT CELL RESEARCH AND THE INTERNATIONAL PIGMENT CELL SOCIETY JUN 2005, vol. 18, no. 3, June 2005 (2005-06), pages 160-166, XP002472113 ISSN: 0893-5785 the whole document	1-6,8-18
X	CINATL J ET AL: "SODIUM VALPROATE INHIBITS IN VIVO GROWTH OF HUMAN NEUROBLASTOMA CELLS" ANTI-CANCER DRUGS, RAPID COMMUNICATIONS, OXFORD, GB, vol. 8, no. 10, November 1997 (1997-11), pages 958-963, XP000997406 ISSN: 0959-4973 abstract	1-3,5-11
X	HESS-STUMPP H: "Histone deacetylase inhibitors and cancer: from cell biology to the clinic" EUROPEAN JOURNAL OF CELL BIOLOGY, WISSENSCHAFLICHE VERLAGSGESELLSCHAFT, STUTTGART,, DE, vol. 84, no. 2-3, 9 March 2005 (2005-03-09), pages 109-121, XP004954663 ISSN: 0171-9335 page 117, left-hand column, paragraph 2 - paragraph 3; table 3 page 118, right-hand column, paragraph 2	1-11
T	DEUBZER HEDWIG E ET AL: "Histone deacetylase inhibitor Helminthosporium carbonum (HC)-toxin suppresses the malignant phenotype of neuroblastoma cells." INTERNATIONAL JOURNAL OF CANCER. JOURNAL INTERNATIONAL DU CANCER 15 APR 2008, vol. 122, no. 8, 15 April 2008 (2008-04-15), pages 1891-1900, XP002472114 ISSN: 1097-0215 the whole document	1-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/061206

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1(in part)-11(in part)
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 1-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.1

Claims Nos.: 1(in part)-11(in part)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/061206

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005117930 A	15-12-2005	NONE	
US 2006229236 A1	12-10-2006	CA 2471957 A1	17-07-2003
		EP 1458746 A2	22-09-2004
		WO 03057722 A2	17-07-2003
		JP 2005517683 T	16-06-2005