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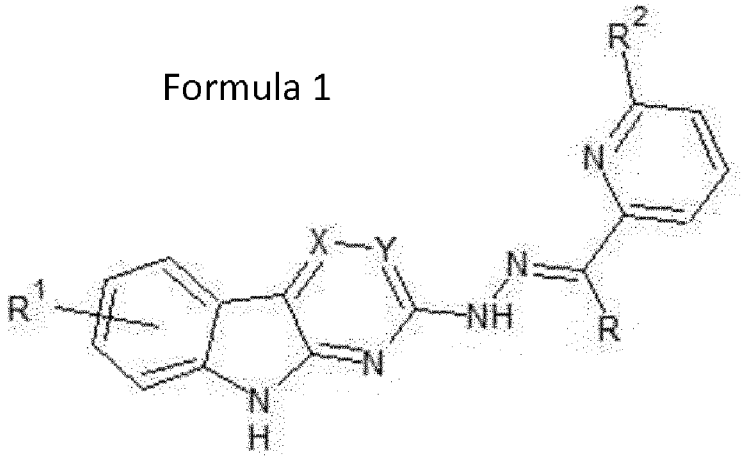
(54) Title: PHARMACEUTICAL COMPOSITION COMPRISING INDOLE DERIVATIVES, PROCESS FOR PREPARATION AND USE THEREOF

Formula 1



(57) Abstract: The invention provides well-defined and stable pharmaceutical compositions comprising indole derivatives of general formula 1, a process for the preparation of di-hydrochloride salts comprising a high content of the pharmacologically active isomer suitable for industrial production, and use of these in pharmaceutical compositions. The invention further provides a method for use of said compounds for the treatment of cancer. The invention also provides methods to use these compounds in conjunction with other therapies commonly used for treating cancer diseases.

Title

Pharmaceutical composition comprising indole derivatives, process for preparation and use thereof.

Field of the invention

The present invention relates to an improved and stable pharmaceutical composition of indole derivatives, comprising a high content of the pharmacologically active isomer thereof. The present invention also relates to a method for the treatment of cancer by use of the compositions and to a process for its preparation. The invention further relates to enabling large scale synthesis of the pharmacologically active compounds.

Background of the invention

Indole derivatives and pharmaceutically acceptable salts thereof are disclosed in WO 2012/128689 and WO 2014/046589 in form of mixtures of cis/trans isomers (Z/E isomers) at the N-methylidene entity. These compounds are useful in the treatment of solid cancers. The anti-cancer effect is believed to be based on the iron-chelating property of the compounds. Since the rate of isomerization at physiological conditions seemed to be substantial it was presumed that the pharmacological effect of the isomers was substantially similar or even the same.

Eshba *et al*., discloses N-(1-pyridine-2-yl-methylidene)-N-(9H-1,3,4,9-tetraza-fluoren-2-yl)-hydrazine derivatives as antiviral and anti-cancer agents, wherein only one compound show cytotoxic activity. It is desirable for a pharmaceutical composition to be well-defined, in particular of its pharmacologically active constituents. It is therefore essential that if a compound exists in two isoforms, the more active isomer of said compounds has to be dominant in the pharmaceutical composition thereof. In addition, a pharmaceutical composition should be sufficiently stable allowing it to be stored for an extended period of time without noticeable change of its constitution.

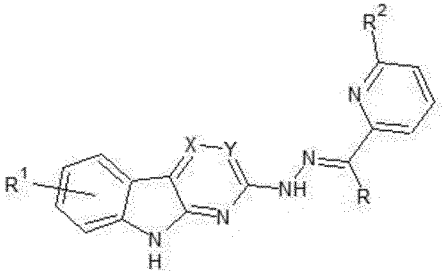
New and effective anticancer drugs need to be developed for patients that suffer from cancer. Drug development over all is associated with a lot of difficulties until a final product is reached. Initially a promising compound is identified and experimentally tested in different *in vitro* models, and after that preclinical studies are initiated most often by the use of different mouse

models. Until this point, only small amounts of the compound need to be synthesized, and the purity requirements are lower than those required in clinical studies conducted in humans. There are many steps in drug development that are critical, e.g., identifying and isolating the active compound, investigating whether a particular isomer is more potent than the other, further have a permissible degree of purity, stability, and also that said compound can be manufactured in large scale. These are not trivial steps and many promising compounds/drugs fail to reach the market due to manufacturing problems as described above.

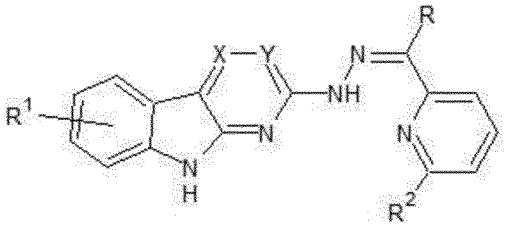
Summary of the invention

The present invention is based on the insight that the mixture of E and Z forms of Formula 1 can be transferred into the E-form of their di-hydrochloride salts of high steric purity.

Formula 1



E-form



Z-form

A first object of the present invention is to provide well-defined and stable pharmaceutical compositions comprising a high content of the pharmaceutically active isomer (E) of compounds or a pharmaceutically acceptable salt thereof represented by general formula 1, wherein:

R is H or methyl or methylene substituted by C_1-C_4 straight or branched alkyl,

R^1 is selected from the group consisting of H, C_1-C_4 straight or branched alkyl, methoxy, methoxy substituted with from one to three fluorine, bromine, halogen;

R^2 is H or C_1-C_4 straight or branched alkyl;

X is CH or N;

Y is CH or N, and

wherein at least 95% by weight (w/w) of the pharmacologically active compound or pharmaceutically acceptable salt thereof is in the form of the E-isomer, as defined in present claim 1.

The pharmaceutical compositions are intended to be used in the treatment of cancer. In one aspect at least 96%, or 97%, or 98%, or at least 98.5% by weight of said compound is in the E-form. In yet another aspect at least 99%, preferably at least 99.5%, most preferably at least 99.8% by weight of the pharmacologically active compound is in the form of the E-isomer. Ideally 100% by weight of said compound is in the form of the E-isomer. The pharmaceutical composition of the present invention may also further comprise at least one pharmacologically acceptable excipient and/or carrier.

According to a preferred embodiment of the invention the compound of the general Formula 1 may be additionally substituted by C_1-C_4 straight or branched alkyl at one of positions 6, 7, 8, 9 of the mono-, di- or tri-azacarbazolyl not substituted by R^1.

Preferred compounds of general Formula 1, as well as 1a and 1b, are listed in Table 1.

In one embodiment R and R^1 are CH_3, and R^2 is H. Preferably R is CH_3 and R^1 is 6-CH_3, and R^2 is H. More preferably X and Y are N.

In another embodiment R is CH_2CH_3, R^1 is CH_3 and R^2 is H. Preferably R is CH_2CH_3, R^1 is 6-CH_3 and R^2 is H. More preferably X and Y are N.

In yet another embodiment R is $CH_2C(CH_3)_3$, R^1 is CH_3 and R^2 is H. Preferably R is $CH_2C(CH_3)_3$, R^1 is 6-CH_3 and R^2 is H. More preferably X and Y are N.

Most preferred compounds of the present invention are compounds A, B and C (See Table 1).

In one embodiment, the pharmaceutical composition of the present invention comprises a pharmacologically active compound of general Formula 1 in the form of a pharmaceutically acceptable salt in crystalline form. The salt may be any salt suitable for stabilization of the free base of Formula 1, i.e., acidic salts, such as for example chlorides, nitrates and sulfates. The salt may be a mono- or di salt. Preferably, the salt is a mono or di-hydrochloride salt. Most preferably a di-hydrochloride salt.

The excipient(s) may be any of mannitol, glucose, sucrose or other suitable sugar derivatives. In a preferred embodiment the excipient is D-mannitol. The concentration of D-mannitol may be in the range of 0.5-20% (w/v). Preferably the concentration is in the range of 1.0-15% (w/v) by weight. More preferably the concentration is in the range of 3-10% (w/v). Most preferably the

concentration is in the range of 4-6% (w/v). The concentration of D-mannitol is in another aspect more preferred to be about 5% (w/v).

Table 1. Exemplary compounds of the invention

Compound	R	R^1	R^2	X	Y
A	CH_3	6-CH_3	H	N	N
B	CH_2CH_3	6-CH_3	H	N	N
C	$CH_2C(CH_3)_3$	6-CH_3	H	N	N
D	CH_3	7-Cl	H	N	N
E	CH_3	6-Cl	H	N	N
F	CH_3	8-OCH_3	H	N	N
G	CH_3	8-OCF_3	H	N	N
H	CH_3	9-Br	H	N	N
I	CH_3	8-Cl	H	N	N
J	CH_3	8-CH_3	H	N	N
K	H	6-CH_3	H	CH	CH

The present invention further provides a process for preparing the pharmaceutical composition described above. The process comprises the following steps:

i. providing a solution of a compound of general formula 1 as a free base,

ii. reacting the solution with hydrochloric acid in ethanol in sufficient amounts to form a compound of general formula 1b, i.e., a di-hydrochloride salt, and wherein the di-hydrochloride salt precipitates spontaneously;

iii. stripping the precipitate comprising the di-hydrochloride salt obtained in step (ii) of solvent,

iv. dissolving the precipitate comprising the di-hydrochloride salt of step (iii) in an aqueous solvent, optionally comprising a pharmaceutically acceptable excipient, and

v. freeze drying the mixture thereby obtaining a lyophilized powder or cake.

The solvent for the free base of general formula 1 may for example be methanol. The stripping of the precipitate i.e., step (iii) may for example be in *vacuo* made by means of an air or inert gas bleed.

The amount of the E-isomer is in the same ranges as for the pharmaceutical composition described above.

In one embodiment, the aqueous solvent is water. Preferably sterile water.

The excipient(s) can be as described above. The order of dissolving the precipitate is not limiting in the process and may be changed. The precipitate may for example be in solid form, mixed with the excipient in for example solid form, and added to an aqueous solvent under stirring. Or, the excipient may be dissolved in an aqueous solution to which the solid precipitate is added and dissolved under stirring.

A further object is to provide a pharmaceutical formulation for injection or infusion in form of an aqueous solution of said storage-stable pharmaceutical composition.

By reconstituting the lyophilized powder of step (v) in an aqueous solvent, for example water for injection (WFI), a pharmaceutical formulation is obtained.

The concentration of the pharmacologically active compound may be in the range of 0.05 to 40 mg/ml. In one embodiment the concentration of the pharmacologically active compound is in the range of 0.1 to 30 mg/ml. More preferably the pharmacologically active compound may be in the range of 0.5-20 mg/ml. Even more preferably the pharmacologically active compound may be in the range of 0.75-10 mg/ml. The concentration of said pharmacologically active compound may most preferably be about 1 mg/ml.

The pH of the formulation is below 4. The pH of said formulation depends on the concentration of the pharmacologically active compound and is usually in the range of 0.5-4. For example a formulation having a concentration of 1 mg/ml of the pharmacologically active compound has a pH in the range of 2-3.

The reconstitution may be performed in one or several steps such as dissolving the lyophilisate by adding a first amount solvent, thereafter adding solvent to a desired final concentration.

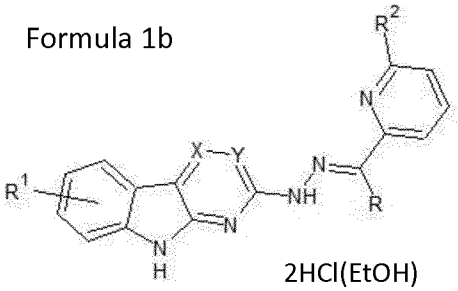
The aqueous solvent for reconstituting the lyophilized powder comprising said pharmaceutically active compound may also comprise a pharmacologically acceptable excipient as described above.

Another object of the present invention is to provide a method for alleviating, reducing or treating cancer in a subject by using the pharmaceutical composition of the invention, alone or in combination with another anticancer treatment.

The administration route of the pharmaceutical formulation may be by infusion or injection. However, any suitable route for administration of the formulation or composition may be used. The formulation or composition may be administered for example intra-arterial, intramuscular, intra-pleural, oral, rectal, enteral, intra-lesional or intra-tumoral, and intrathecal administration.

Another object of the present invention is to provide a precipitate exemplified by general Formula 1b,

Formula 1b



2HCl(EtOH)

wherein at least 95% by weight (w/w) of the pharmacologically active compound of general Formula 1b is in the form of the E-isomer.

The amount of the E-isomer may be in same the ranges as for the pharmaceutical composition described above.

The compound of general Formula 1b is a precipitate of the indole derivative of Formula 1, wherein the substitutions R, R^1, R^2, X and Y are as defined above for Formula 1. Preferred compounds of general Formula 1b are listed in Table 1. Most preferred compounds of general Formula 1b are substituted as compounds A, B and C in Table 1.

Another object of the present invention is to provide a process for preparing the precipitate comprising said compounds or pharmaceutically acceptable salts described above, said process corresponds to process steps i) to iii) described above for the pharmaceutical composition.

In one aspect, the di-hydrochloric acid in ethanol (i.e., step ii) is added in two steps, wherein 1.0 – 1.15 equivalents of hydrochloric acid in ethanol is added in the first step and 2.0 to 2.5 equivalents of hydrochloric acid in ethanol is added in the second step. Alternatively, addition may be performed in one step or several steps. The salt precipitates spontaneously in step (ii).

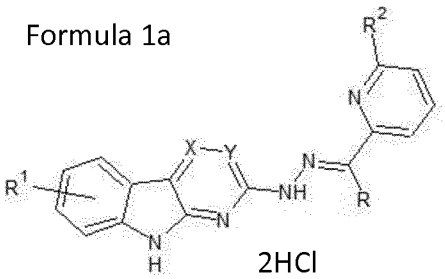
The precipitate described above can also be used in a pharmaceutical composition.

The precipitate can be used directly or after drying before further processing to a lyophilisate.

The ethanol content of said precipitate is in the range of 2-15% by weight of said precipitate. Preferably in the range of 4-13% or, 9-11% by weight of said precipitate. In one embodiment the amount ethanol is 10.4-10.6% by weight of said precipitate.

The present invention further provides a lyophilisate comprising a compound of general Formula 1a,

Formula 1a



2HCl

wherein at least 95% by weight (w/w) of the pharmacologically active compound of general Formula 1a is in the form of the E-isomer. The amount of the E-isomer may be in the same range as for the pharmaceutical composition described above.

The compound of general Formula 1a is a di-hydrochloride salt of the indole derivatives described above for Formula I.

Most preferred compounds are substitutes as described for formula 1 and 1b above.

The present invention further provides a process for preparing said lyophilisate, the process comprise the following steps:

a) dissolving a precipitate of general Formula 1b in an aqueous solvent,

b) filtering the resulting solution,

c) freeze drying the solution of step b) to obtain a lyophilisate comprising a compound of general Formula 1a.

In one aspect the precipitate may be dissolved in the aqueous solvent under stirring in step a). The process is further described in the detailed description.

The precipitate of step a) may be substituted as any of the compounds described for Formula 1 or 1b. In another aspect the precipitate may comprise one or a combination of the described compounds. In yet another aspect separate precipitates comprising different compounds of the present invention may be mixed.

The aqueous solvent may further comprise at least one pharmacologically acceptable excipient. The excipient and concentration of excipient may be as described above.

The resulting solution of step b) may preferably be filtered through at least one sterile filter, in some embodiments the resulting solution of step b) is filtered through two sterile filters. The resulting solution may for example be recovered in a sterile bulk before step c). The solution of step b) may also be filled into vials suitable for freeze drying.

Yet another object of the present invention is to provide a precipitate or lyophilisate as described above for use in a pharmaceutical composition.

The pharmaceutical composition (i.e., the lyophilisate) and precipitate of the present invention are stable for at least 12 months in room temperature. Preferably the pharmaceutical composition (i.e., the lyophilisate) and precipitate are stable for at least 24 months in room temperature.

Yet another object of the present invention is to provide a pharmaceutical composition, i.e., a lyophilisate comprising said compounds for use in treating cancer.

In one aspect, the lyophilisate of the present invention may comprise only one pharmacologically active compound of the present invention such as for example compound A2, B2 or C2. In another aspect the lyophilisate of the present invention may comprise a combination of compounds of the present invention. In yet another aspect, the lyophilisate comprising said compounds or pharmaceutically acceptable salts of the present invention may comprise at least one of the compounds of the present invention in combination with at least one other pharmacologically active compound for use in cancer treatment.

The compounds of the present invention may be administered separately or as a mixture. The compounds may further be administered at the same time or prior to or after another medicament or anticancer treatment.

The pharmaceutical composition, precipitate or the formulation described above may for example be used for prevention or in the treatment of a disease or disorder characterized by pathologically proliferating cells.

The pharmaceutical formulation may be suitable for infusion or injection by reconstituting said composition in an aqueous solvent. Preferably the formulation is used for infusion.

The final concentration of the pharmacologically active compound may be in the range of 0.5-30 mg/ml.

The pharmaceutical composition and formulation may have a pH in the range of 0.5-4. Preferably the pH is in the range of 1-3. As mentioned above, the pH depends on the concentration of the pharmaceutically active compound, and for example the pH for a 1 mg/ml formulation is in the range of 2-3.

The pharmaceutical composition or formulation may further comprise a co-therapeutic agent.

Preferably, the pharmaceutical composition and formulation of the present invention is used for treating cancer.

The cancer may be a solid, liquid and haematological tumor.

Further, the medicament, pharmaceutical formulation, composition, precipitate or lyophilisate described above may be used in combination with another anticancer treatment such as chemotherapy, immunological or immunomodulating therapy, hormone therapy, surgical removal of the tumour, photodynamic therapy, laser therapy, hyperthermia, cryotherapy, angiogenesis inhibition, radiation therapy, or a combination of these.

The present invention further provides a method for treating a disease or disorder characterized by pathologically proliferating cells, such as cancer, in which an effective amount of a pharmacologically active compound of the present invention is administered to a subject in need of such treatment.

The effective amount of said pharmacologically active compound or compounds varies among individuals and cancer form. For example the amount is about 0.1-10 mg/kg body weight,

preferably 0.5-5 mg/kg and more preferably 1-4 mg/kg body weight. The total dose given to a subject may be in the range of 5-800 mg, depending on the subject's condition and cancer form and independent of the weight of said subject. In one aspect the dose administered to a subject is in the range of 30 to 300 mg. The dose can be even lower when given in combination with another cancer treatment as exemplified below.

In another aspect, the invention provides a method for the treatment of cancer described above in combination with another anticancer treatment.

The different embodiments described above can be combined with each other or used separately.

The details of one or more embodiments of the invention are set forth in the detailed description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the appended claims, hereby incorporated by reference.

Brief description of Figures

The following figures are illustrative of aspects of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Fig. 1 shows the synthetic route for synthesis of the precipitate (A1) of compound A, and the salt formation step of the precipitate to the corresponding salt, i.e., lyophilisate (A2).

Fig. 2a depicts a HPLC-chromatogram showing 99.8% purity of compound A1, and Fig. 2b illustrates the E-isomer structure of compound A1 confirmed by X-ray chromatography.

Fig. 3 show dose-response curves for compound A in various cell lines.

Figs. 4a-d show dose-response curves for compound A, B and C in HCT116-cells (A), and in HepG2-cells, RKO-cells, HeLa-cells, CEM-cells and THP-1 cells for compound A (b), for compound B (c) and for compound C (d).

Detailed description

It is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since

the scope of the present invention will be limited only by the appended claims and equivalents thereof.

All references cited are incorporated herein by reference in their entirely and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

The present invention is best understood by reference to the following definitions, the Figures and exemplary disclosure provided herein.

In this specification, the compound of general Formula 1 is intended to include any pharmaceutically suitable precipitate, solvate, salt or prodrug thereof.

In this specification the term precipitate means the di-hydrochloride ethanol co-crystal compounds, or the di-hydrochloride ethanolate or the di-hydrochloride ethanol solvate obtained by precipitation e.g., the product of the precipitation step in reaction 4 in Fig. 1. The compounds may be a precipitate of any compound of formula 1 of the present invention.

In this specification the term pharmaceutically acceptable compounds comprise precipitates, solvates and lyophilisates of the compounds described in the present specification.

In this specification the term "isomer" refers to compounds that have the same composition and molecular weight but differ in physical and/or chemical properties. Such substances have the same number and kind of atoms but differ in structure. The structural difference may be in constitution (geometric isomers) or in an ability to rotate the plane of polarized light (stereoisomers). The term "stereoisomer" refers to isomers of identical constitution that differ in the arrangement of their atoms in space.

In this specification, unless otherwise stated, the term "pharmaceutically acceptable excipient" means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In this specification, unless otherwise stated, the term "pharmaceutically active compound" encompasses any substance that will produce a therapeutically beneficial pharmacological response when administered to a host, including both humans and animals.

In this specification the term "administering" or "administration" means providing a drug to a subject in a manner that is pharmacologically useful.

In this specification, unless otherwise stated, the term "cytotoxic compound" refers to a compound that has the ability of arresting the growth of, or killing, cells, i.e., having high cytotoxic activity.

In this specification, unless otherwise stated, the term "derivative" refers to a compound formed from the original structure either directly, by a chemical reaction of the original structure, or by a "modification" which is a partial substitution of the original structure, or by design and *de novo* synthesis. Derivatives may be synthetic, or may be metabolic products of a cell or an *in vitro* enzymatic reaction.

In this specification the term "cancer" is meant to mean any malignant neoplastic disease, i.e. any malignant growth or tumor caused by abnormal and uncontrolled cell division. The term "cancer" is in particular meant to include both solid, localized tumors, and non-solid cancer forms. For example said cancer forms may be selected from the group consisting of leukemia (ALL, AML, CLL, CML, CMML), T-cell leukemia, multiple myeloma, ovarian carcinoma, prostate cancer, cervix adenocarcinoma, squamous cell carcinoma, breast cancer, colorectal cancer, small bowel cancer, anal cancer, gastric cancer, kidney cancer, malignant melanoma cancer of the renal pelvis and ureter, urethral cancer, bladder cancer, liver cancer, appendix cancer, pancreas cancer, lung cancer, cancer of the oesophagus, lip/oral cavity cancer, nasal cancer, larynx cancer, brain/central nervous system cancer, skin cancer, thyroid and thymus cancer, sarcoma, head and neck cancer, Non-Hodgkin lymphoma (NHL), Hodgkin lymphoma, and pseudomyxoma peritonei.

The present invention provides a process for preparing a pharmaceutical composition which is favorable to the E-isomer. Single crystal X-ray confirmed that the E-isomer was predominant in the solid state.

By using the process of the present invention a well-defined and stable pharmaceutical composition comprising at least 95% by weight (confirmed by HPLC, See Fig. 2) of the pharmaceutically active compound (E-isomer), is obtained.

EXAMPLES

Example 1 Synthesis of compound A

In the first experiments compound A (free base) was diluted in acetone/acetylate/acetone nitrile, the E-isomer but not the Z-isomer was soluble in this solvent combination, and was easily filtered of. The final E-isomer content by using this solvent combination was about 92%. The described solvent combination worked well during small scale production but not for scaling up production due to high amounts of solvent needed. Therefore, synthesis of compound A based on the synthesis of 1, 2, 4-triazino[5,6-b]indole derivatives described by Kgokong, *et al*., 2005 was developed by the inventors (See Fig.1). The inventors developed a procedure using methanol (MeOH) as solvent, and hydrochloric acid in ethanol (HCl/EtOH) as carrier of HCl (EtOH also serves as an anti-solvent). In the subsequent development of the scaling up process the reaction volume efficiency was improved. Moreover, a suitable method for conversion of the free base (A) to the final hydrochloride precipitate (A1) on a large scale was also developed (See Fig. 1, Examples 1 and 2). The free base (A) was not soluble in MeOH alone, but upon addition of about 1 equivalent of HCl/EtOH a clear solution was obtained.

Due to observed disulphide species, the reaction may be performed under nitrogen to avoid air oxidation. The wet cake produced by reaction step 1 may also be dried in vacuo, or the wet cake may be further processed without prior drying. By drying in vacuo the generation of impurities is minimalized, since impurities may be generated during air-vented drying. Reacting the product compound of reaction step 2 with a slight excess of 2-acetylpyridine (1.5 eq.) in ethanol (20 mL/gram compound) at 50˚C gave product formation, but too slow conversion (~8%) after 5 hrs.

Fig. 1 shows reaction steps 1-3 of the synthesis of compound A (mixture of E and Z isomers; IUPAC systematic name: 2-[(1E,Z)-1-(2-{6-methyl-5H-[1,2,4]triazino[5,6-b]indole-3-yl}hydrazine-1-ylidene)ethyl]-pyridine).

Step 1. To an aqueous suspension of 7-methylisatin (4.75 kg, 29.5 mol) was added 2.85 kg (31.3 mol) of thiosemicarbazide and 6.15 kg (44.5 mol) of potassium carbonate. The stirred mixture was heated under reflux for 3 hrs, then cooled to room temperature. Acetic acid (100%, 3.3 kg, 55.0 mol) was slowly added until a pH of 7.1 had been reached. The suspension was filtered on a pressure filter and the filter cake washed with water (19.4 kg) to obtain 7.6 kg of wet 6-methyl-2H,3H,5H-[1,2,4]triazino[5,6-b]indole-3-thione.

Step 2. The wet filter cake from the preceding step corresponding to about 4.6 kg of dry 6-methyl-2H,3H,5H-[1,2,4]triazino[5,6-b]indole-3-thione was suspended in 57.1 kg of hydrazine monohydrate and the mixture stirred at 89 °C for 18 hrs. The reaction mixture was cooled to room temperature and the product isolated by centrifugation, washed with water (15.9 kg) and ethanol (18.4 kg), and drained at 1450 RPM). The wet filter cake (7.8 kg corresponding to 3.8 kg dry weight) of 3-hydrazinyl-6-methyl-5H-[1,2,4]triazino[5,6-b]indole was transferred back to the cleaned reactor and dried under vacuum.

Step 3. To the dried 3-hydrazinyl-6-methyl-5H-[1,2,4]triazino[5,6-b]indole from Step 2 was added water (76.85 kg), acetic acid (100%, 6.70 kg, 111.6 mol) and 2-acetylpyridine (10.75 kg, 88.7 mol). The mixture was stirred for 3 hrs at 48.5°C, cooled to room temperature and NaOH (27%, 6.3 kg, 110 mol) slowly added to reach pH 7.0 while maintaining the temperature between 20 and 25°C. The mixture was stirred for further 1 ¼ hrs at this temperature and the product isolated by centrifugation. After washing with a mixture of water (7.3 kg) and ethanol (5.8 kg) the cake was drained at 1450 RPM, then dried in a vacuum oven at 47°C for 66 hrs to yield 5.82 kg of the title compound in form of a beige/greenish solid material.

Step 4 in Fig. 1 shows the synthesis of Compound A1, the ethanol co-crystal of compound A *(IUPAC systematic name: 2-[(1E)-1-(2-{6-methyl-5H-[1,2,4]triazino[5,6-b]indole-3-yl}hydrazine-1-ylidene)ethyl]-pyridine di-hydrochloride)*

To 2-[(1E,Z)-1-(2-{6-methyl-5H-[1,2,4]triazino[5,6-b]indole-3-yl}hydrazine-1-ylidene)ethyl]-pyridine) (5.80 kg) was added ethanolic HCl (12.4 kg, 1.05 equiv.) and the mixture stirred at 28-30°C for half an hour until a clear solution was obtained. The solution was filtered and additional ethanolic HCl (28.95 kg, 2.45 equiv.) was added at 25°C over 1 h and 40 min under stirring. During the first addition of 1.05 equiv. HCl/EtOH the majority of the Z-isomer present transforms to the E-isomer and some monohydrochloride salt is formed. The di-hydrochloride salt precipitates spontaneously by the addition of 2.45 equiv. HCl in EtOH. Molarity determination of HCl in EtOH by titration with 0.1 M NaOH phenolohthalein indicator was calculated to be about 1.1 to 1.4 M HCl. Stirring was continued at the same temperature for 15 min and ethanol (45.8 kg) added. The so formed suspension was cooled to about 0 to -5°C and stirred for 1 h. The product isolated by centrifugation was washed with ethanol (0 to 5°C, 45 kg), then drained at 1450 RPM. The cake was dried in vacuum at 37°C for 42 hrs to yield 7.57 kg

of the title compound (about 108% on residual solvent-free basis or 98% based on mono-EtOH, di-hydrochloride as a yellow to orange solid.

The ethanol co-crystalline di-hydrochloride precipitate obtained has a content of ethanol from about 2% to 20% by weight.

Reaction step 5 in Fig. 1 illustrates the formation of the freeze dried composition comprising a compound of general formula 1a.

Analysis of Isomer content by HPLC

During the process development, analysis of compound A and compound A1 caused analytical problems due to e.g. sample instability, poor solubility, isomerisation, HPLC, etc. Therefore a more robust HPLC method was developed by the inventors based on an XBridge C18, 3.5 µm, 150 x 4.6 mm column. The problem was further solved by using 2% formic acid in MeOH as diluent, and switching from uncoated standard HPLC sample vials to coated (silanized) vials from Agilent.

Agilent 1200/1260 chromatographic system or equivalent was used.

When using the acidic HPLC to analyse compound A it was found that ~7% was in the form of the Z-isomer (sample preparation in 0.1% TFA/H_2O). After 2 days the same sample was re-analysed showing ~2% of the Z-isomer, and the beginning of hydrolysis to compound A1 (~1% detected). This showed that acidic conditions (pH in the range of 1-4) transforms the unwanted Z-isomer to the desired E-isomer. When the subsequent salt formation (reaction step 4) was performed (using HCl in ethanol), the isomeric content was lowered to < 0.5%. This means that a relatively large content of the unwanted isomer (such as 5%) can be allowed of compound A, B or C since it is being converted to the desired isomer upon addition of HCl in ethanol. The addition of HCl in ethanol forms a di-hydrochloride precipitate (such as compounds A1, B1 and C1).

HPLC purity

HPLC purity was calculated as 100% - total impurities. All peaks below 0.05% and peaks present in the matrix are excluded from the calculations. The content of each impurity was calculated as percentage of the total peak area (area %). Total impurities are the sum of impurities ≥ 0.05%.

Impurities

The final result of each impurity is the average of four results. Total impurities are reported as the sum of impurities ≥ 0.05%.

Residual solvents

Analysis of compound A1 showed that it is a di-hydrochloride ethanol co-crystal composition (precipitate). The theoretical ethanol content of compound A1 is 10.6%, which is consistent with the formation of an ethanol co-crystal (precipitate) as described above.

During the process development of compositions comprising compound A it was surprisingly shown that the di-hydrochloride ethanol co-crystal (e.g., A1) is less hygroscopic and significantly more stable towards hydrolysis and degradation of isomeric purity.

It was concluded that high levels of ethanol could be tolerated in the drug substance (precipitate) since it is removed during the subsequent freeze drying, which is part of the manufacturing process of the final drug product (lyophilisate).

The methanol levels showed to be relatively high; typically methanol contents of composition A1 was 1.4 – 1.8%. Prolonged drying cycles did not significantly decrease the methanol content. However, as in the case with ethanol, the subsequent freeze drying cycle used during manufacturing of the final drug product (e.g., A2), efficiently removes the methanol down to levels below the ICH Q3B guideline.

Conclusion

Based on the fact that both the ethanol and methanol levels are well below the ICH Q3C guideline in the final drug product and given that this is carefully monitored, it was concluded that the higher levels could be allowed in the drug substance (i.e., precipitate of compound A1). All other limits stated in the specification are within Ph. Eur or USP standards.

Identification

The identity of a sample was based on a visual inspection of the main peak of a sample preparation and the main peak of the sample preparation for identification. Compound A1 is represented by a single peak in the chromatogram (See Fig. 2a).

Example 2. Stability

The stability study of the di-hydrochloride ethanol co-crystal precipitate and of the lyophilized di-hydrochloride salt was conducted in accordance with the International Conference on Harmonizations (ICH) guideline Q1A (R2) Stability Testing of New Drug Substances and Products. All analytical instruments used to analyze the stability samples during the study are qualified in compliance with current cGMP.

The stability study consists of two parts, one long-term- (5°C, 24, 36 months) and one accelerated study (25°C/60 % RH, 6 months).

The di-hydrochloride ethanol co-crystal precipitate of compound A (A1) was packed in heat sealed double polyethylene bags inside a heat sealed foiled laminate pouch placed in a closed HDPE container. The samples were stored at the long term condition 5°C and at the accelerated condition 25°C/60%RH. The appearance was yellow to orange solid during the whole test period. Analysis performed due to the X-Ray powder diffraction result for 25°C/60%RH sample which had an unexpected low level of crystallinity. The level of crystallinity has no direct effect on the quality or stability of the drug substance but is controlled as part of the development work. The 36 month stability data obtained are summarized in table 2a below.

Table 2b shows the stability data for the di-hydrochloride ethanol co-crystal precipitate at 25°C and 60% RH over a period of 6 months. The appearance was yellow to orange solid during the whole period.

Conclusion

The present composition comprising compound A1 is stable for at least 24 months (Table 2a). During this period no significant breakdown of compound A1 occurred at either 2-8°C or 25°C/60%RH (6 months). It is suggested that the composition of compound A1 should be stored and transported at 2-8°C. However, 24 hours of storage at temperatures up to 25°C should be of no concern.

Example 3. Manufacture of a pharmaceutical composition of the ethanol co-crystal precipitate of 2-[(1)-1-(2-{6-methyl-5H-[1,2,4]triazino[5,6-b]indole-3-yl}hydrazine-1-ylidene)ethyl]-pyridine di-hydrochloride.

A multiple of 225.6 mg of ethanol co-crystal precipitate of mainly 2-[(1E)-1-(2-{6-methyl-5H-

[1,2,4]triazino[5,6-b]indole-3-yl}hydrazine-1-ylidene)ethyl]-pyridine di-hydrochloride (A1) (corresponds to 160 mg free base, A) was dissolved in a solution of mannitol (500 mg) in water for injection (Ph. Eur., 10 ml), the solution was sterilized by filtration through two 0.2 µm filters and filled into a corresponding number of sterilized vials, then freeze dried (obtaining a salt of compound A2).

Table 2a

Time (months)		**0**	**1**	**3**	**6**	**9**	**12**	**18**	**24**	**36**
RRT 0.92-0.93	≤1.0	0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.07	<0.05	0-07
RRT 1.13	≤1.0	<0.05	<0.05	<0.06	<0.06	<0.05	0.05	0.05	0.05	<0.05
RRT 1.23-1.24	≤1.0	0.05	0.05	0.06	0.06	0.05	0.06	0.06	0.06	0.06
RRT 1.39	≤1.0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.05	<0.05	<0.05
RRT 1.47-1.51	≤1.0	0.17	0.10	0.10	0.36[2]	0.09	<0.05	0.06	0.05	0.06
Total impurities	≤2.0	0.26	0.15	0.16	0.42	0.14	0.10	0.29	0.16	0.18
Water content (% w/w)		2.53	2.34	2.18	2.50	2.88	2.65	2.73	3.37	2.46

[2] The relative area for the impurity at RRT = 1.47 – 1.51 is higher than expected. The sample preparation and HPLC analysis was repeated by another analyst, which confirmed the result. Fluctuating peak area for this impurity was observed during the test method validation.

The inventors developed a new freeze drying process since the ordinary methods used by prior art required more than 300 hrs of drying. The new method is more aggressive and outlined in table 3 below.

Table 2b					
Time (months)		**0**	**1**	**3**	**6**
RRT 0.92-0.93	≤ 1.0	0.05	< 0.05	0.05	0.07
RRT 1.12	≤ 1.0	< 0.05	< 0.05	< 0.05	0.05
RRT 1.24	≤ 1.0	0.05	0.05	0.06	0.05
RRT 1.38	≤ 1.0	< 0.05	< 0.05	0.05	< 0.05
RRT 1.49-1.51	≤ 1.0	0.17	0.10	0.06	0.36[2]
Total impurities	≤ 2.0	0.26	0.16	0.22	0.52
Water content (% w/w)		2.53	2.41	2.30	2.45

[2] The relative area for the impurity at RRT = 1.47 – 1.51 is higher than expected.

The sample preparation and HPLC analysis was repeated by another analyst, which confirmed the result. Fluctuating peak area for this impurity was observed during the test method validation.

By having max negative pressure and relatively high temperature, annealing the temperature as in Table 3, the freeze drying step was decreased to 19 hrs.

Contact with metallic surfaces was avoided. Ethanol and minor amounts of methanol present were removed by the freeze drying process.

The vials were crimp sealed under nitrogen and stored at 5°C; no degradation was seen after storage for 24 months.

Glucose and mannitol were evaluated as excipients, both alone and in combination with NaCl. The best result regarding solubility, texture of the lyophilised cake and suppression of impurity formation was obtained with 5% (w/v) mannitol as additive. A higher degree of collapse of the freeze-dried cake was observed with glucose as bulking agent. Addition of NaCl caused solubility problems since the increase in pH generated by NaCl decreased the solubility of compound A2.

The lyophilised powder for reconstitution and injection (corresponding to 160 mg free base of compound A) were stored at conditions 2-8°C up to 24 months. The appearance was yellow to orange freeze dried cake during the whole test period and after reconstitution yellow to orange solution without visible particles.

Table 3

Step type	Temperature (T °C)	Time (h)	Vacuum (mbar)
Shelves	5	/	/
Freezing step	5	0.30	/
Freezing ramp	-45	0.50	/
Freezing step	-45	4	/
Freezing ramp (Annealing)	-25	1	/
Freezing step (Annealing)	-25	2	/
Freezing ramp (Annealing)	-45	1	/
Freezing step	-45	4	/
Chamber Vacuum	-45	/	0.200
Primary drying	-45	0.10	0.200
Primary drying ramp	25	3	0.200
Primary drying step	25	XX*	0.200
Secondary drying ramp	25	10	max
End of cycle			

Analysis performed due to the X-Ray powder diffraction result for 25°C/60%RH had an unexpected low level of crystallinity. The level of crystallinity has no direct effect on the quality or stability of the drug substance but is controlled as part of the development work. The reconstitution time was up to 3 minutes. No bacterial growth was detected and the sterility of the product was not influenced during the 24 months period in room temperature. The stability data obtained is summarized in Table 4a below.

Table 4a

Time (months)	0	1	3	6	12	18	24
pH	1.6	1.6	1.6	1.5	1.6	1.6	1.7
Water content (%)	0.33	0.41	0.47	0.37	0.53	0.43	0.39
Assay (% w/w)[1]	97.8	98.4	95.9	97.3	93.9	94.6	94.2
Total impurities (%)	0.7	0.5	0.3	0.4	0.2	0.3	0.34
Any individual purity (%)	0.4	0.3	0.1	0.1	0.1	0.1	0.10
Specified impurity* (%)	<0.5	<0.05	<0.05	0.08	0.08	0.10	0.09
Z-isomer (%)	0.1	0.1	0.1	0.1	<0.05	<0.05	0.05
RRT 0.92-0.93							
RRT 1.13	0.05	<0.05	<0.05	<0.05	<0.05	0.05	<LOQ
RRT 1.23-1.24	0.06	0.05	0.08	0.05	0.06	0.05	0.05
RRT 1.39							
RRT 1.47-1.51	0.37	0.32	0.05	<0.05	<0.05	0.05	0.10

*Hydrolysis impurity 3-Hydrazinyl-6-methyl-5H-[1,2,4]triazinol[5,6-b]indole

The LOQ is 0.05%, peaks < than LOQ was recorded as <0.05%

The lyophilised powder for reconstitution and injection (corresponding to 160 mg free base of compound A) were stored at accelerated conditions 25°C/60%RH (See Table 4b). The appearance was yellow to orange freeze dried cake during the whole test period and after reconstitution yellow to orange solution without visible particles. Analysis performed due to the X-Ray powder diffraction result for 25°C/60%RH had an unexpected low level of crystallinity. The level of crystallinity has no direct effect on the quality or stability of the drug substance but is controlled as part of the development work. The reconstitution time was up to 3 minutes. No bacterial growth was detected and the sterility of the product was not influenced during the 24 months period in room temperature. Surprisingly, the lyophilisate showed to be

stable at least 24 months in room temperature. The stability data obtained is summarized in Table 4b below.

Table 4b

Time (months)	0	1	3	6	12	18	24
pH	1.6	1.6	1.6	1.5	1.6	1.5	1.6
Water content (%)	0.33	0.39	0.55	0.45	0.59	0.49	0.45
Assay (% w/w)[1]	97.8	97.2	95.4	98.3	94.6	93.9	94.5
Total impurities (%)	0.7	0.3	0.7	0.4	0.2	0.20	0.36
Any individual purity (%)	0.4	0.1	0.2	0.1	0.1	0.1	0.10
Specified impurity* (%)	<0.5	0.1	<0.05	0.08	0.08	0.10	0.10
Z-isomer (%)	0.1	<0.05	0.1	0.1	0.10	<0.05	0.05
RRT 0.92-0.93							
RRT 1.13	0.05	<0.05	<0.05	<0.05	<0.05	nd	<LOQ
RRT 1.23-1.24	0.06	0.05	0.08	0.06	0.06	0.06	0.06
RRT 1.39							
RRT 1.47-1.51	0.37	0.13	0.05	<0.05	<0.05	nd	0.09

*Hydrolysis impurity 3-Hydrazinyl-6-methyl-5H-[1,2,4]triazinol[5,6-b]indole

The LOQ is 0.05%, peaks < than LOQ was recorded as <0.05%. The pH should be in the range of 0.5-4, in the example above the concentration is about 16 mg/ml and the pH is in the range of

1.3 to 2.3 and water content below 1%. The Z-isomer is preferably less than 2%, however the inventors surprisingly found that acidic conditions favour the E-isomer.

The lyophilisate comprising compound A2 surprisingly showed to be less soluble in water after lyophilisation than prior to. Due to this, a structural investigation was conducted and this study showed that compound A2 changes its crystalline form during freeze-drying. The new crystalline form was less soluble in water, which explains the difference in solubility between the di-hydrochloride ethanol co-crystal precipitate (A1) and the di-hydrochloride salt (A2). Experiments indicated that this exhaustion of precipitates induced the change of morphous form. Results from the experiments also showed that the excipient (D-Mannitol) does not have any impact on the formation of the new morphic form. The best result concerning formation of impurities and texture of the freeze dried cake was obtained with the freeze drying cycle described in Table 3 and the mannitol content set at 5%.

Example 4. Preparation of a pharmaceutical formulation.

It was found that compound A2 could be formulated in aqueous media to supress formation of by-products up to 24 hrs at 1 mg/ml. Also, it is understood that the pH is of significance for the stability of compound A2 in aqueous media with the best stability at pH around 1-4, higher concentration of the substance results in lower pH. 1 mg/ml of said aqueous solution has a pH about 2-3.

The resulting compound A2 is formulated as a sterile lyophilised powder, and a solution for injection or infusion was prepared by dissolving the lyophilized powder described above in an aqueous solvent such as water for injection. Each vial contains an amount pharmacologically active compound corresponding to 160 mg free base (A) prepared from a solution of 225.6 mg drug substance (A1), and 5% mannitol (w/v). The lyophilisate may be reconstituted in 10 ml aqueous solvent, and thereafter diluted to 1 mg/ml in an aqueous solvent optionally comprising a pharmacologically acceptable excipient, preferably 5% mannitol (w/v), for infusion.

Example 5.

Synthesis of compound B; 2-[(1E)-1-(2-{6-methyl-5H-[1,2,4]triazino[5,6-b]indole-3-yl}hydrazin-1-ylidene)propyl]pyridine

1-(pyridin-2-yl)propan-1-one (35 mg, 0.26 mmol) was dissolved in a water –acetic acid mixture (20:1, 10 mL) then 3-hydrazinyl-6-methyl-5H-[1,2,4]triazino[5,6-b]indole (50 mg, 0.23 mmol)

was added. The reaction mixture was stirred for 2 hours at 50°C. After evaporating the solvents, a dark green solid was obtained (70 mg). LC shows pure product with an isomer ratio of 95:5.

Example 6

Synthesis of compound C; 2-(3,3-dimethyl-N-{6-methyl-5H-[1,2,4]triazino[5,6-b]indole-3-yl}butanehydrazonoyl)pyridine

3,3-dimethyl-1-(pyridin-2-yl)butan-1-one (46 mg, 0.26 mmol) was measured in a water – acetic acid mixture (20:1, 10 mL) then 3-hydrazinyl-6-methyl-5H-[1,2,4]triazino[5,6-b]indole (48 mg, 0.23 mmol) was added. The reaction mixture was stirred overnight at 50°C. After evaporating the solvents, a greenish yellow solid was obtained (78 mg). LC showed pure product with an isomer ratio of 92:8.

Example 7

The conversion of compound B1 to its di-hydrochloride (B2) was prepared by the following procedure:

2-[(1E)-1-(2-{6-methyl-5H-[1,2,4]triazino[5,6-b]indole-3-yl}hydrazin-1-ylidene)propyl]pyridine (30 mg, 0.09 mmol) was suspended in methanol (0.6 mL), then HCl in ethanol (1.04 equiv. 1.25 M, 75 µL) was added dropwise. After all the solid was dissolved, more HCl in ethanol (2.08 equiv. 1.25 M, 150 µL) and ethanol (0.6 mL) was added. A light brown precipitate appeared. The suspension was kept at -10°C for 3 hours, then the solid was filtered, washed with cold ethanol and dried. The product was a bright yellow solid (10 mg). LC shows only one isomer, the minor isomer is not detected after converting the product to its HCl salt.

Example 8

The conversion of compound C1 to its di-hydrochloride C2 was prepared by the following procedure: 2-[(1E)-1-(2-{6-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl}hydrazin-1-ylidene)propyl]-pyridine (30 mg, 0.09 mmol) was suspended in methanol (0.6 mL), then HCl in ethanol (1.04 equiv. 1.25 M, 75 µL) was added dropwise. After all the solid was dissolved, more HCl in ethanol (2.08 equiv. 1.25 M, 150 µL) and ethanol (0.6 mL) was added. The product did not precipitate immediately, only after the suspension was kept at -10°C for 3 hours. The solid was filtered, washed with cold ethanol and dried. The product was a bright yellow solid (20

mg). LC shows only one isomer (E), the minor isomer (Z) is not detected after converting the product to its HCl salt.

Characterization

Single crystal X-ray showed that the E-isomer is predominant in the solid state.

Single Crystal X-ray was performed at SARomics Biostructures AB, Sweden. Crystals of compound A1 measuring about 100 x 30 µm were picked up in standard cryo loops of the kind normally used for protein crystals, immersed in paraffin oil and flash-cooled in liquid nitrogen. Data were collected at 100 K at station I911-3 of MAX-lab (λ = 0.9198 Å), equipped with a 225 mm mar CCD detector. The beam size was 50 x 50 µm. The x-ray results confirm that compound A1 is the E-hydrazone isomer. The predicted structure is shown in Fig. 2b, where N stands for nitrogen atoms, H for hydrogen atoms, CL for chloride atoms, and H_2O for water molecules.

All testing was performed using reference standard, and, all analyses are in agreement with the proposed structure.

Conclusion

A water content of 4-7% in the starting material of compound A was tolerable, even though the product compound A2 readily hydrolyses in aqueous solvents.

There was no trace of isomer in the mother liquor, showing that the applied precipitation condition converts the Z-isomer to the target E-isomer. Preferably, the salt formation should be performed within hours since the product is acid sensitive.

The composition development work was initiated with the aqueous solvent stability testing and excipient evaluation discussed above. Based on these results, the composition development continued by optimising the composition (i.e. drug substance- the ethanol co-crystal precipitate - concentration and type and quantity of excipient) with regards to the effect on impurity formation and solubility of the drug product (i.e., the di-hydrochloride salt, for example compound A2). As a result of the optimisation the amount of the free base (compound A) per vial was increased from 100 to 160 mg.

Example 9 Cytotoxic activity

Cytotoxic activity expressed as survival Index, (IC50), by compound A is shown in various cell lines (Fig. 3) and primary cultures of human tumors (Table 5). The Fluorometric Microculture

Cytotoxicity Assay (FMCA), (Lindhagen *et al*., 2008), was used for measurement of the cytotoxic effect of the compounds in various cell lines and primary cultures of human tumors. Cells were seeded in the drug-prepared 384-well plates using the pipetting robot Precision 2000 (Bio-Tek Instruments Inc., Winooski, VT). The plates were incubated for 72 h and then transferred to an integrated HTS SAGIAN Core System consisting of an ORCA robot (Beckman Coulter) with CO_2 incubator (Cytomat 2C, Kendro, Sollentuna, Sweden), dispenser module (Multidrop 384, Titertek, Huntsville, AL), washer module (ELx 405, Bio-Tek Instruments Inc), de-lidding station, plate hotels, barcode reader (Beckman Coulter), liquid handler (Biomek 2000, Beckman Coulter) and a multipurpose reader (FLUOstar Optima, BMG Labtech GmbH, Offenburg, Germany) for automated FMCA.

Different cell lines (e.g. CCRF-CEM T-cell leukemia, RPMI-8226 multiple myeloma, A2780 ovarian carcinoma, FaDu head & neck cancer (squamous cell carcinoma tumor), HT29 colorectal cancer, MCF7 breast cancer, and HL-60 leukemia cells) as well as panels of primary human tumor cell cultures (Table 5) were analyzed (colon, gastric, kidney, appendix, small bowel and pancreas cancer, as well as pseudomyxoma peritonei). Results show broad anti-cancer activity of compound A, as exemplified in the effect-concentration graph (Fig. 3).

Example 10

The inventors also set out to characterize activity of the compounds A, B and C in cell lines representing cancer of different origin. The specific assays used and the conclusions from the mechanistic evaluation have previously been described in detail (Zhang *et al*. 2014). Compounds A, B and C (See Fig. 4) were evaluated for cytotoxicity expressed as survival index (SI) in six human tumor cell lines using the cell based fluorometric micro culture cytotoxicity assay (FMCA) as previously described in detail (Lindhagen *et al*, 2008). The method is based on measurement of fluorescent fluorescein, generated from hydrolysis of FDA by viable cells with intact plasma membrane. The fluorescence is proportional to the number of intact viable cells.

Material and methods

Cell culture

The cell lines were cultivated in the respective cell medium recommended by the provider. The medium was supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-

glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin (all from Sigma-Aldrich). The cell line was cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Measurement of cytotoxic activity

FMCA analysis in brief, 2500 cells per well were seeded into 384-well microplates and incubated over night before treatment with compounds. Compounds were added using acoustic liquid transfer (Echo 550, LabCyte). The plates were incubated at 37°C for 72 h, and then washed and FDA was added to the wells followed by 50 min of incubation at 37°C. The fluorescence, which is proportional to the number of living cells in each well, was measured at 485/520 nm in a Fluoroskan instrument (Labsystems, GMI, Ramsey, MIN). Cell survival is presented as Survival Index (SI), defined as the fluorescence value in the compound-treated wells analysed as percentage of the value in the control wells, with blank values subtracted. Quality criteria included a signal/blank ratio >10 and a coefficient of variation (CV) in control and blank wells <30%. Graph Pad Prism (San Diego, California, USA). All experiments were performed twice, and each concentration was evaluated in quadruplicates in each experiment. The compounds (A, B and C) were diluted DMSO, 5 mM.

Table 5. IC_{50} in panels of different primary human tumor cell cultures

Disease	No of patients analyzed	IC_{50} µM
PMP*	50	9.4
Colorectal**	25	11
Gastric	9	6.9
Renal	13	164
Mesothelioma	7	12
Appendix	4	21
Small bowel	1	5.4
Ovarian	30	5.7
Pancreas	1	6.0

**Pseudomyxoma Peritonei, ** Colorectal cancer, surgical specimens obtained from maximal cytoreductive surgery and peritinectomies*

Results and discussion

The tested compounds (A, B and C) showed strong activity on a wide range of cancer cell lines, see Table 6 and Fig. 4. The cell lines were selected to cover a wide range of cancer types, representing both haematological and solid tumors (Table 6).

From these results, it is clearly shown that compounds A, B and C are effective against several different tumor cell lines including colon carcinoma, cervix adenocarcinoma, hepatocellular carcinoma, acute lymphoblastic leukemia and acute monocytic leukemia.

From the results presented here, it is clearly shown that compounds A, B and C are effective against several different tumor cell lines including colon carcinoma, cervix adenocarcinoma, hepatocellular carcinoma, acute lymphoblastic leukemia and acute monocytic leukemia.

Table 6. IC_{50} for compounds A, B and C in six human tumor cell lines.

Cell line	**Origin**	**IC_{50} Compound A**	**IC_{50} Compound B**	**IC_{50} Compound C**
HCT116	Colon carcinoma	≈ 1 µM	≈ 1 µM	≈ 1 µM
RKO	Colon carcinoma	< 250 nM	< 250 nM	< 250 nM
HeLa	Cervix Adenocarcinoma	≈ 20 µM	≈ 20 µM	≈ 10 µM
HepG2	Hepatocellular Carcinoma	< 250 nM	< 250 nM	< 250 nM
CCRF-CEM	Acute lymphoblastic leukemia	< 250 nM	< 250 nM	< 250 nM
THP-1	Acute Monocytic Leukemia	< 250 nM	< 250 nM	< 250 nM

Although particular embodiments have been discussed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims that follow. In particular, it is contemplated by the inventor that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims.

References

Eshba *et al*. *Synthesis of some substituted-1,2,4-triazino[5,6-b]indole derivatives as potential antiviral and anticancer agents.* Pharmazie Vol. 42, No. 10, 1987; 664-666.

Lindhagen E, Nygren P, Larsson R (2008) The fluorometric microculture cytotoxicity assay. Nature Protocls 3: 1364-1369.

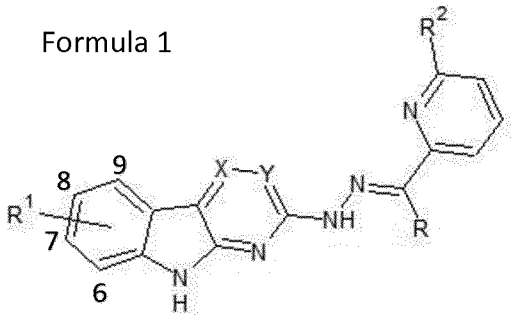
Kgokong JL, Smith PP, Matsabisa GM(2005) Bioorg Med Chem. 13(8):2935-42).

Zhang X *et al*. (2014) Induction of mitochondrial dysfunction as a strategy for targeting tumor cells in metabolically compromised microenvironments. Nature communications 5:3295.

Claims

1. A pharmaceutical composition for use in treating cancer comprising a pharmaceutically active compound of general formula 1, or a pharmaceutically acceptable salt thereof,

Formula 1



wherein,

R is H or methyl or methylene substituted by C_1-C_4 straight or branched alkyl,

R^1 is selected from the group consisting of H, C_1-C_4 straight or branched alkyl, methoxy, methoxy substituted with from one to three fluorine, bromide, halogen;

R^2 is H or C_1-C_4 straight or branched alkyl;

X is CH or N;

Y is CH or N, and wherein at least 95% by weight (w/w) of the pharmacologically active compound, or pharmaceutically acceptable salt thereof is in the form of the E-isomer.

2. The pharmaceutical composition according to claim 1, wherein the pharmacologically active compound is a salt in crystalline form.

3. The pharmaceutical composition according to claim 2, wherein the salt is a di-hydrochloride.

4. The pharmaceutical composition according to any of claims 1-3, further comprising a pharmaceutically acceptable excipient in the concentration of 0.1-10% (w/v).

5. The pharmaceutical composition according to any of claims 1-4, having a stability of at least 12 months.

6. A process for preparing the pharmaceutical composition according to any of claims 1-5 comprising the following steps:

i. providing a solution of a compound of general formula 1 as a free base,

ii. reacting the solution with hydrochloric acid in ethanol in sufficient amounts to form a compound of general formula 1 to a di-hydrochloride salt, wherein the di-hydrochloride salt precipitates spontaneously;

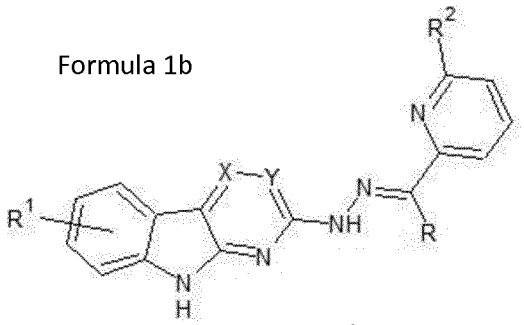
iii. stripping the precipitate comprising the di-hydrochloride salt obtained in step (ii) of solvent,

iv. dissolving the di-hydrochloride salt of step (iii) in an aqueous solvent, optionally comprising a pharmaceutically acceptable excipient, and

v. freeze drying the mixture thereby obtaining a lyophilized powder or cake.

7. A precipitate comprising a compound of general formula 1b,

Formula 1b



.2HCl.EtOH

wherein R is H or methyl or methylene substituted by C_1-C_4 straight or branched alkyl,

R^1 is selected from the group consisting of H, C_1-C_4 straight or branched alkyl, methoxy, methoxy substituted with from one to three fluorine, bromide, halogen;

R^2 is H or C_1-C_4 straight or branched alkyl;

X is CH or N;

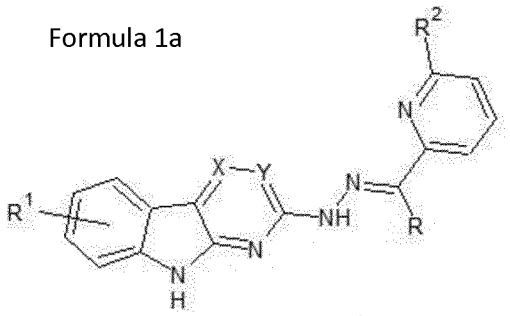
Y is CH or N, and wherein at least 95% by weight (w/w) of compound 1b is in the form of the E-isomer.

8. A process for preparing the di-hydrochloride salt precipitate according to claim 7, comprising the steps i) to ii) and optionally step iii) of claim 6.

9. The di-hydrochloride salt precipitate according to claim 7 wherein residual ethanol is in the range of 2-20% by weight of the di-hydrochloride salt precipitate.

10. A lyophilisate comprising a compound of general formula 1a,

Formula 1a



.2HCl

wherein R is H or methyl or methylene substituted by C_1-C_4 straight or branched alkyl,

R^1 is selected from the group consisting of H, C_1-C_4 straight or branched alkyl, methoxy, methoxy substituted with from one to three fluorine, bromide, halogen;

R^2 is H or C_1-C_4 straight or branched alkyl;

X is CH or N;

Y is CH or N, and wherein at least 95% by weight (w/w) of compound 1a is in the form of the E-isomer.

11. A process for preparing the lyophilisate according to claim 10 comprising the following steps:

i. dissolving a di-hydrochloride salt precipitate of formula 1b in an aqueous solvent, optionally comprising a pharmaceutically acceptable excipient, and

ii. freeze drying the mixture thereby obtaining a lyophilized powder or cake.

12. The di-hydrochloride salt precipitate according to claim 7 or 9 and the lyophilisate according to claim 10 for use in a pharmaceutical composition.

13. A pharmaceutical formulation suitable for infusion prepared by reconstituting the pharmaceutical composition, or pharmaceutically acceptable salts according to any of claims 1-5, 7, 9 and 10 in an aqueous solvent at a final concentration in the range of 0.5-30 mg/ml.

14. The pharmaceutical formulation according to claim 13, wherein the pH is in the range of 0.5-4.

15. The pharmaceutical composition, pharmaceutically acceptable salts or formulation according to any of claims 1-5, 7, 9, 10, 12, 13 and 14, for use in treating cancer.

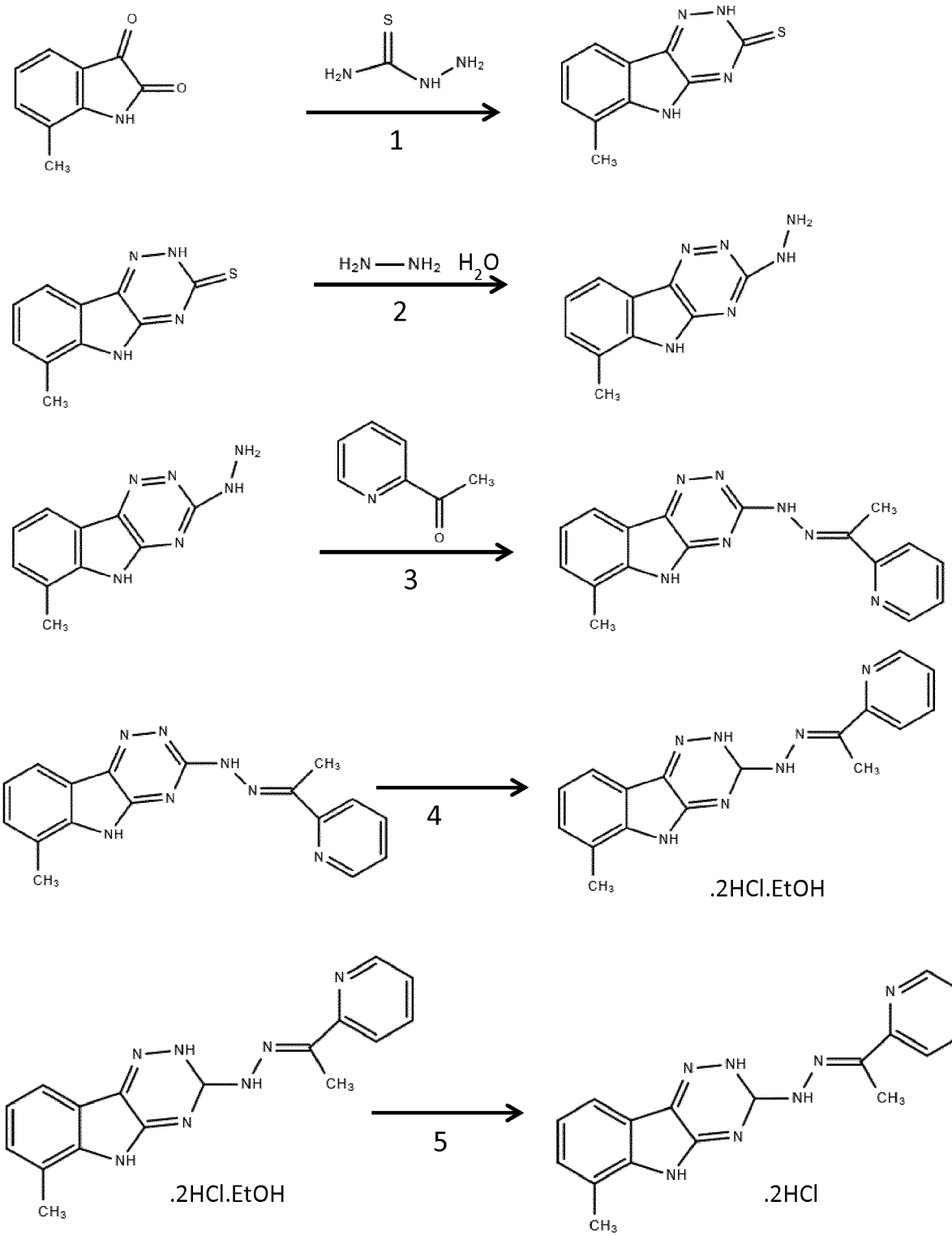
16. The pharmaceutical composition, pharmaceutically acceptable salts or formulation according to claim 15, wherein the cancer is a solid, liquid or heamatological tumor.

17. A method for treating cancer in a subject in which an effective amount of a pharmaceutical formulation or composition comprising the compound, or pharmaceutically acceptable salts thereof according to any of claims 1-5, 7, 9, 10, 12, 13 and 14 is administered to a subject in need of such treatment.

18. The method for the treatment of cancer according to claim 17 in combination with another anticancer treatment.

19. The method for treating cancer according to any of claims 17 or 18 wherein the effective dose is in the range of 0.01-10 mg/kg body weight, preferably 0.1-5 mg/kg body weight and more preferably 1-4 mg/kg body weight.

Fig. 1



Received at EPO via Web-Form on Dec 10, 2016

Fig.2A

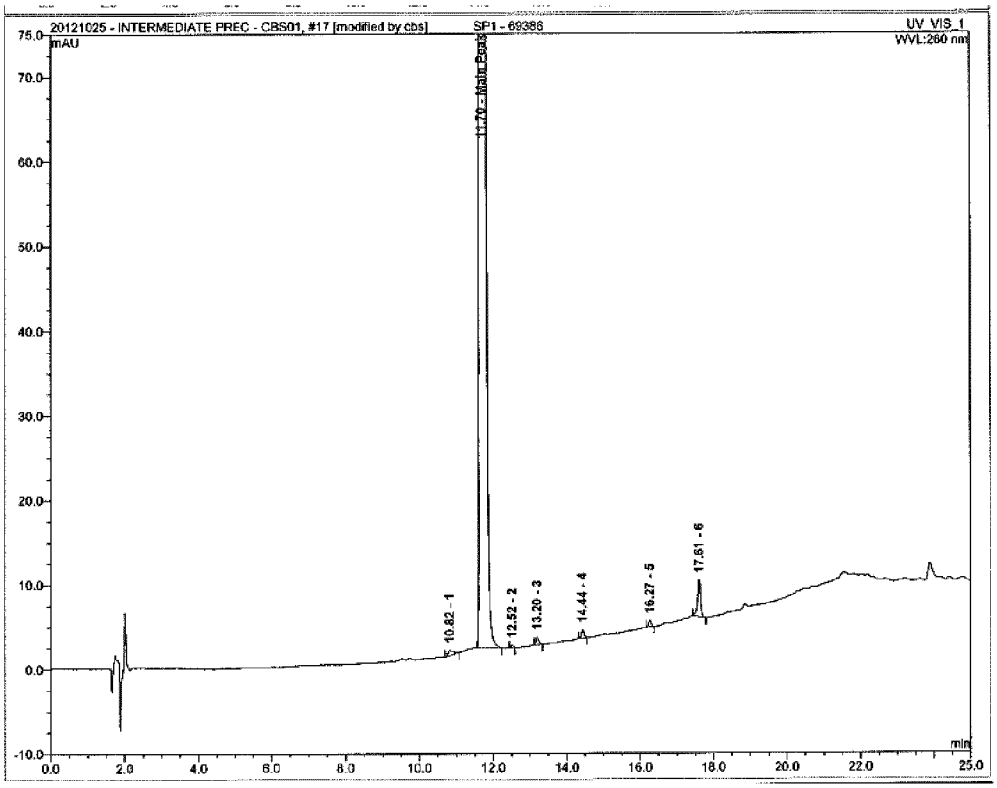

20121025 - INTERMEDIATE PREC - CBS01, #17 [modified by cbs]
SP1 - 69386
UV_VIS_1
WVL:260 nm
mAU
min
10.82 - 1
11.70 - Main Peak
12.52 - 2
13.20 - 3
14.44 - 4
16.27 - 5
17.61 - 6

Fig. 2B

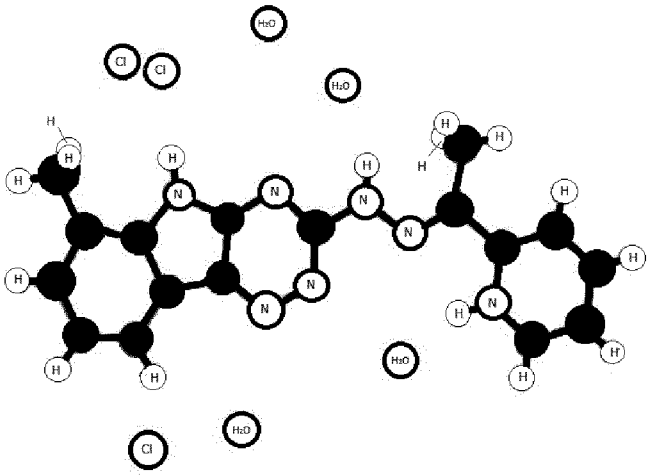

H2O
Cl
Cl
H2O
H2O
H2O
Cl

Fig. 3

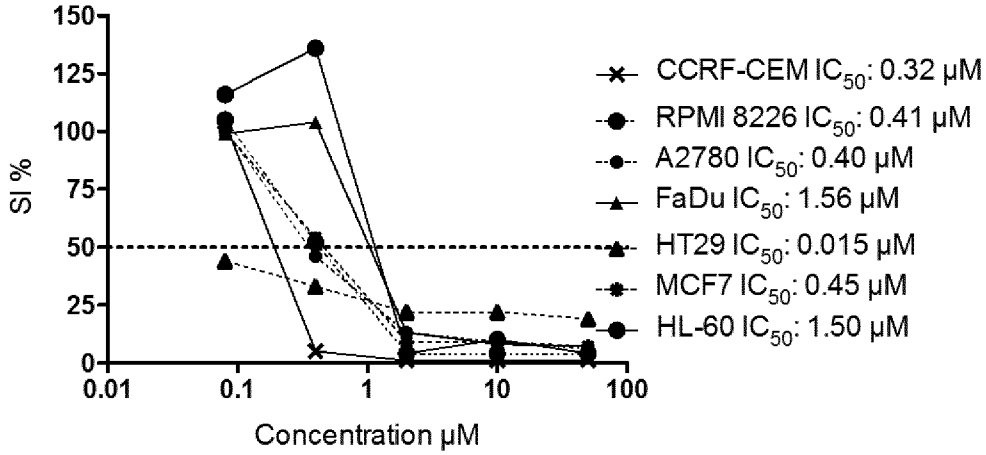
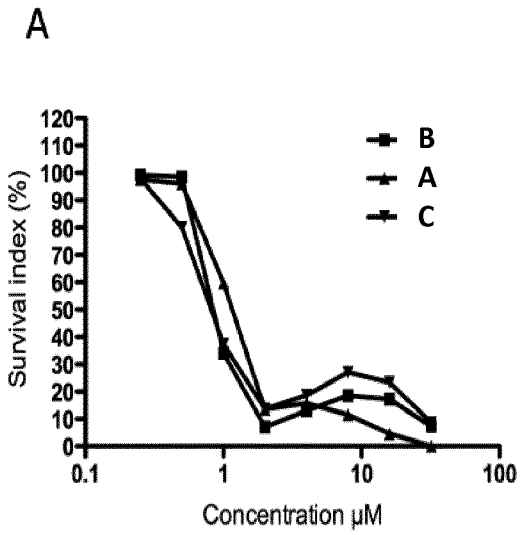


Fig. 4



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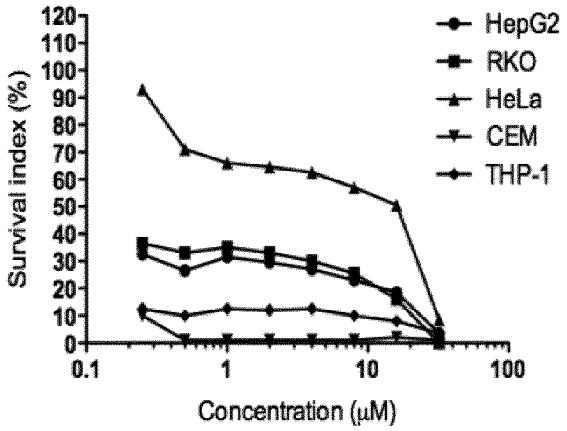
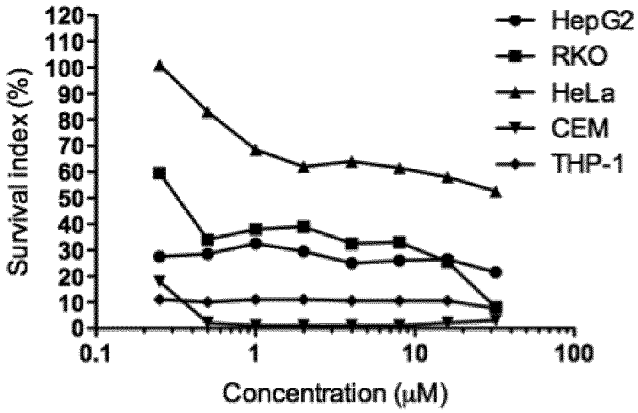
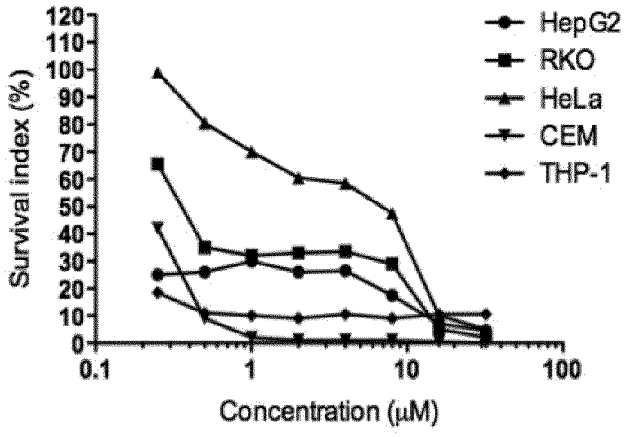
B



C



D



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/025175

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D471/04 A61K31/4439 A61P35/00
ADD.

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)
C07D

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 practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/046589 A1 (VIVOLUX AB [SE]) 27 March 2014 (2014-03-27) examples 2,8	1-19
X	WO 2012/128689 A1 (VIVOLUX AB [SE]; LINDER STIG [SE]; FRYKNAES MAARTEN [SE]; LARSSON ROLF) 27 September 2012 (2012-09-27) the whole document	1-19

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

[X] See patent family annex.

* Special categories of cited documents :

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"E" earlier application or patent 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
3 April 2017	12/04/2017

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, Fax: (+31-70) 340-3016	Baston, Eckhard

1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/025175

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ESHBA N H ET AL: "Synthesis of some substituted 1,2,4-triazino[5,6-b]indole derivatives as potential antiviral and anticancer agents", DIE PHARMAZIE: AN INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES, GOVI VERLAG PHARMAZEUTISCHER VERLAG GMBH, DE, vol. 42, no. 10, 1 January 1987 (1987-01-01), pages 664-666, XP009029588, ISSN: 0031-7144 the whole document -----	1-19
X	XIAONAN ZHANG ET AL: "Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments", NATURE COMMUNICATIONS, vol. 5, 18 February 2014 (2014-02-18), XP055264964, DOI: 10.1038/ncomms4295 the whole document -----	1-19

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/025175

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014046589 A1	27-03-2014	AU 2013318672 A1	19-03-2015
		CA 2884832 A1	27-03-2014
		CN 104662022 A	27-05-2015
		EA 201590315 A1	31-08-2015
		EP 2900667 A1	05-08-2015
		HK 1206352 A1	08-01-2016
		JP 2015529244 A	05-10-2015
		KR 20150058441 A	28-05-2015
		SG 11201502209P A	28-05-2015
		US 2015259349 A1	17-09-2015
		WO 2014046589 A1	27-03-2014
WO 2012128689 A1	27-09-2012	AU 2012231814 A1	26-09-2013
		BR 112013024211 A2	20-12-2016
		CA 2830081 A1	27-09-2012
		CN 103547268 A	29-01-2014
		EA 201391286 A1	31-03-2014
		EP 2688569 A1	29-01-2014
		IL 228411 A	29-12-2016
		JP 6064215 B2	01-02-2017
		JP 2014508804 A	10-04-2014
		KR 20140017619 A	11-02-2014
		SG 193494 A1	30-10-2013
		SG 10201605083S A	30-08-2016
		US 2014073645 A1	13-03-2014
		WO 2012128689 A1	27-09-2012
		ZA 201307036 B	28-05-2014

(19)中华人民共和国国家知识产权局

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(51)Int.Cl.
C07D 471/04(2006.01)
A61K 31/4439(2006.01)
A61P 35/00(2006.01)

权利要求书3页 说明书18页 附图4页

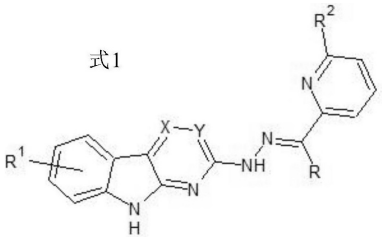
(54)发明名称

含有吲哚衍生物的药物组合物、其制备方法和用途

(57)摘要

本发明提供了包含通式1的吲哚衍生物的定义明确且稳定的药物组合物、制备包含适合于工业生产的高含量药理学活性异构体的二盐酸盐的方法、以及这些在药物组合物中的用途。本发明还提供将所述化合物用于治疗癌症的方法。本发明还提供了将这些化合物与通常用于治疗癌症疾病的其他疗法联合使用的方法。

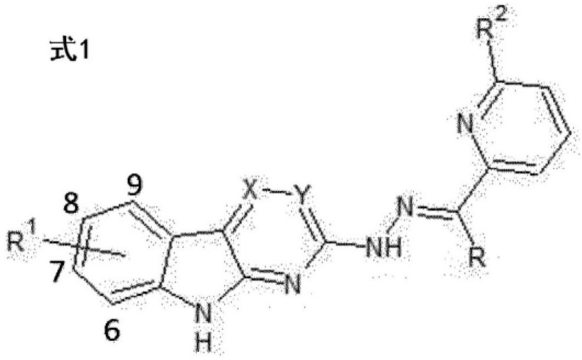
式1



CN 108431003 A

1.用于治疗癌症的药物组合物,其包含通式1的药理学活性化合物或其药学上可接受的盐,

式1



其中,

R是H、或甲基、或被C_1-C_4直链或支链烷基取代的亚甲基;

R^1选自下组:H、C_1-C_4直链或支链烷基、甲氧基、被一个至三个氟、溴、卤素取代的甲氧基;

R^2是H、或C_1-C_4直链或支链烷基;

X是CH或N;

Y是CH或N,并且,至少95重量%(w/w)的药理学活性化合物或其药学上可接受的盐呈E-异构体的形式。

2.如权利要求1所述的药物组合物,其特征在于,所述药理学活性化合物是结晶形式的盐。

3.如权利要求2所述的药物组合物,其特征在于,所述盐是二盐酸盐。

4.如权利要求1至3中任一项所述的药物组合物,所述药物组合物还包含浓度0.1%至10%(w/v)的药学上可接受的赋形剂。

5.如权利要求1-4中任一项所述的药物组合物,所述药物组合物具有至少12个月的稳定性。

6.一种制备如权利要求1至5中任一项所述的药物组合物的方法,其包括以下步骤:

i.提供游离碱形式的通式1的化合物的溶液,

ii.所述溶液与足量的乙醇中的盐酸反应以使通式1的化合物形成二盐酸盐,其中二盐酸盐自发沉淀;

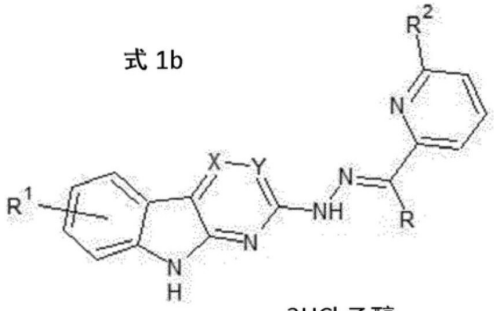
iii.将步骤(ii)溶剂中得到的包含二盐酸盐的沉淀物进行剥离,

iv.将步骤(iii)的二盐酸盐溶解于水性溶剂中,所述水性溶剂任选地包含药学上可接受的赋形剂,以及

v.将混合物进行冷冻干燥,由此获得冻干粉末或饼。

7.一种含有通式1b的化合物的沉淀物,

式 1b



.2HCl.乙醇

其中,R是H、或甲基、或被C_1-C_4直链或支链烷基取代的亚甲基;

R^1选自下组:H、C_1-C_4直链或支链烷基、甲氧基、被一个至三个氟、溴、卤素取代的甲氧基;

R^2是H、或C_1-C_4直链或支链烷基;

X是CH或N;

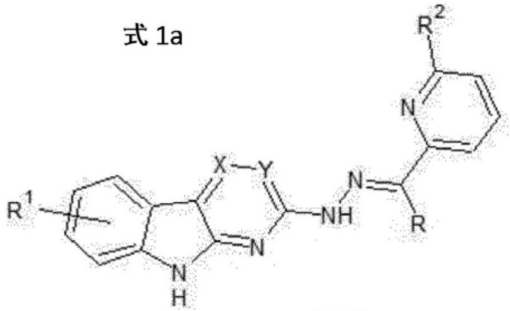
Y是CH或N,并且,至少95重量%(w/w)化合物1b呈E-异构体的形式。

8.一种用于制备如权利要求7所述的二盐酸盐沉淀物的方法,所述方法包括权利要求6的步骤i)至iii)。

9.如权利要求7所述的二盐酸盐沉淀物,其中,残留乙醇占二盐酸盐沉淀物的2-20重量%。

10.一种含有通式1a的化合物的冻干物,

式 1a



.2HCl

其中,R是H、或甲基、或被C_1-C_4直链或支链烷基取代的亚甲基;

R^1选自下组:H、C_1-C_4直链或支链烷基、甲氧基、被一个至三个氟、溴、卤素取代的甲氧基;

R^2是H、或C_1-C_4直链或支链烷基;

X是CH或N;

Y是CH或N,并且,至少95重量%(w/w)化合物1a呈E-异构体的形式。

11.一种用于制备如权利要求10所述的冻干物的方法,其包括以下步骤:

i将通式1b的二盐酸盐沉淀物溶解于水性溶剂中,所述水性溶剂任选地包含药学上可接受的赋形剂,以及

ii.将混合物进行冷冻干燥,由此获得冻干粉末或饼。

12.如权利要求7或9所述的二盐酸盐沉淀物或如权利要求10所述的冻干物在药物组合物中的用途。

13.一种适于输注的药物制剂,其通过将如权利要求1-5、7、9和10中任一项所述的药物

组合物或药学上可接受的盐以0.5-30mg/ml的最终浓度范围在水性溶剂中进行重构来制备。

14.如权利要求13所述的药物制剂,其特征在于,pH为0.5至4。

15.如权利要求1至5、7、9、10、13和14中任一项所述的药学组合物、药学上可接受的盐或制剂,其用于治疗癌症。

16.如权利要求15所述的药学组合物、药学上可接受的盐或制剂,所述癌症是实体肿瘤、液体肿瘤或血液肿瘤。

17.一种用于治疗对象的癌症的方法,其中,将有效量的包含如权利要求1至5、7、9、10、13和14中任一项所述的化合物或其药学上可接受的盐的药物制剂或组合物给予需要该治疗的对象。

18.如权利要求17所述的治疗方法,其与另一种抗癌治疗组合。

19.根据权利要求17或18中任一项所述的治疗癌症的方法,其特征在于,有效剂量为0.01-10mg/kg体重,优选0.1-5mg/kg体重,更优选1-4mg/kg体重。

含有吲哚衍生物的药物组合物、其制备方法和用途

技术领域

[0001] 本发明涉及改进且稳定的吲哚衍生物的药物组合物，包含高含量的其药理学活性异构体。本发明还涉及使用该组合物治疗癌症的方法及其制备方法。本发明还涉及能够大规模合成的药理学活性化合物。

[0002] 发明背景

[0003] 吲哚衍生物及其药学上可接受的盐以在N-亚甲基主体处的顺式/反式异构体(Z/E异构体)混合物的形式公开于WO 2012/128689和WO 2014/046589。这些化合物用于实体癌症的治疗。抗癌效果被认为是基于化合物的铁螯合性质。由于异构化率在生理条件下似乎相当，假定异构体的药理作用是基本相似或甚至相同的。

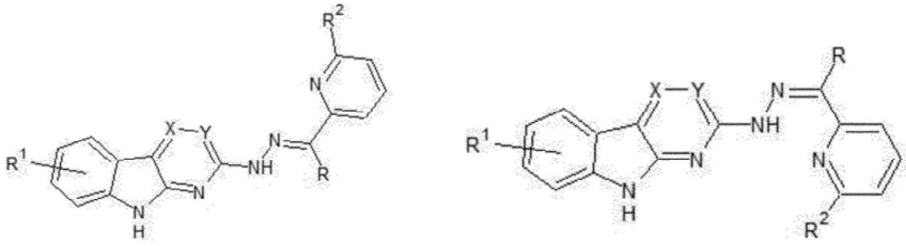
[0004] Eshba等人公开了N-(1-吡啶-2-基-亚甲基)-N-(9H-1,3,4,9-四氮杂-芴-2-基)-肼衍生物作为抗病毒和抗癌剂，其中仅一种化合物显示出细胞毒活性。理想的是药物组合物定义明确，特别是其药理学活性成分。因此，如果化合物以两种同种型存在，那么所述化合物的更具活性的异构体在其药物组合物中必须占优势。此外，药学组合物应当足够稳定，以允许其以长期储存而成分没有显著变化。

[0005] 需要为患癌症的患者开发新型且有效的抗癌药物。在获得最终产品之前，所有药物研发有很多困难。最初，一种有前景的化合物被鉴定出来并在不同的体外模型中进行实验测试，然后通过使用不同的小鼠模型进行临床前研究。直到此刻，仅需要合成少量的化合物，并且纯度要求低于人体临床研究所需的纯度。药物研发中有许多步骤是关键的，例如鉴定和分离活性化合物，研究特定异构体是否比其他异构体更有效，是否还具有允许程度的纯度、稳定性以及所述化合物是否可以大规模制造。这些并非无关紧要的步骤，由于上述的制造问题，许多有希望的化合物/药物未能进入市场。

发明内容

[0006] 本发明基于如下发现：可以将式1的E和Z形式的混合物转化为其高立构纯度(steric purity)的E形式的二盐酸盐。

[0007] 式1

[0008] 

E-形式 Z-形式

[0009] 本发明的第一目的是提供定义明确且稳定的药物组合物，所述药物组合物包含高含量的通式1所述的化合物的药理学活性异构体(E)和其药学上可接受的盐。

[0010] R是H、或甲基、或被C_1-C_4直链或支链烷基取代的亚甲基；

[0011] R^1选自下组:H、C_1-C_4直链或支链烷基、甲氧基、被一个至三个氟、溴、卤素取代的甲氧基;

[0012] R^2是H、或C_1-C_4直链或支链烷基;

[0013] X是CH或N;

[0014] Y是CH或N;并且

[0015] 其中,至少95重量%(w/w)的药理学活性化合物或其药学上可接受的盐呈如权利要求1所限定的E-异构体的形式。

[0016] 药物组合物旨在于在癌症治疗中使用。一方面中,至少96重量%、或97重量%、或98重量%或至少98.5重量%的所述化合物是E形式的。另一方面中,至少99重量%、优选至少99.5重量%、最优选至少99.8重量%的药理学活性化合物是E异构体的。理想地,100重量%的所述化合物是E异构体。本发明的药物组合物还可以包含至少一种药学上可接受的赋形剂和/或载体。

[0017] 根据本发明的优选的实施方式,通式1的化合物还可在未被R^1取代的单-、双-或三-氮杂咔唑基的6、7、8、9位之一上被C_1-C_4直链或支链烷基取代。

[0018] 通式1以及1a和1b的优选化合物如表1所列。

[0019] 在一实施方式中,R和R^1是CH_3,并且R^2是H。优选地,R是CH_3,并且R^1是6-CH_3,R^2是H。更优选X和Y是N。

[0020] 在另一实施方式中,R是CH_2CH_3,R^1是CH_3,并且R^2是H。优选地,R是CH_2CH_3,R^1是6-CH_3,并且R^2是H。更优选X和Y是N。

[0021] 在另一实施方式中,R是$CH_2C(CH_3)_3$,R^1是CH_3,并且R^2是H。优选地,R是$CH_2C(CH_3)_3$,R^1是6-CH_3,并且R^2是H。更优选X和Y是N。

[0022] 本发明最优选的化合物是化合物A、B和C(参见表1)。

[0023] 在一实施方式中,本发明的药物组合物包含结晶形式的药学上可接受的盐形式的通式1的药理学活性化合物。盐可以是适用于稳定式1游离碱的任意盐,即酸性盐,例如,盐酸盐、硝酸盐和硫酸盐。盐可以是单盐(mono salt)或二盐(di salt)。优选地,所述盐是单盐酸盐或二盐酸盐。最优选二盐酸盐。

[0024] 赋形剂可以是甘露醇、葡萄糖、蔗糖或其他合适的糖衍生物中的一种。在一个优选的实施方式中,赋形剂是D-甘露醇。D-甘露醇的浓度范围可以是0.5%至20%(w/v)。优选地,浓度范围为1.0重量%至15重量%(w/v)。更优选地,浓度范围为3%至10%(w/v)。最优选地,浓度范围为4%至6%(w/v)。在另一方面中,D-甘露醇的浓度范围更优选约为5%(w/v)。

表 1. 本发明示例性化合物

[0025]

化合物	R	R^1	R^2	X	Y
A	CH_3	6-CH_3	H	N	N
B	CH_2CH_3	6-CH_3	H	N	N
C	$CH_2C(CH_3)_3$	6-CH_3	H	N	N
D	CH_3	7-Cl	H	N	N
E	CH_3	6-Cl	H	N	N

[0026]

F	CH_3	8-OCH_3	H	N	N
G	CH_3	8-OCF_3	H	N	N
H	CH_3	9-Br	H	N	N
I	CH_3	8-Cl	H	N	N
J	CH_3	8-CH_3	H	N	N
K	H	6-CH_3	H	CH	CH

[0027] 本发明还提供了用于制备如上所述药物组合物的方法。所述方法包括以下步骤：

[0028] i.提供游离碱形式的通式1的化合物的溶液，

[0029] ii.所述溶液与足量的乙醇中的盐酸反应以形成通式1b的化合物，即，二盐酸盐，其中所述二盐酸盐自发沉淀；

[0030] iii.将步骤(ii)溶剂中得到的包含二盐酸盐的沉淀物进行剥离(stripping)，

[0031] iv.将包含步骤(iii)的二盐酸盐的沉淀物溶解于水性溶剂中，所述水性溶剂任选地包含药学上可接受的赋形剂，以及

[0032] v.将混合物进行冷冻干燥，由此获得冻干粉末或饼。

[0033] 用于通式1的游离碱的溶剂可以是例如甲醇。沉淀物的剥离(即，步骤(iii))可以例如通过空气或惰性气体排出而在真空中进行。

[0034] E-异构体的量的范围与上述药物组合物相同。

[0035] 在一实施方式中，水性溶剂是水。优选无菌水。

[0036] 赋形剂可以是如上文所述的。溶解沉淀物的顺序在该过程中不受限制并且可以改变。沉淀物可以是例如，固体形式的、与赋形剂例如以固体形式混合、以及在搅拌下加入至水性溶剂中。或者，赋形剂可以溶解于加入固体沉淀物的水性溶液中，并在搅拌下溶解。

[0037] 另一目的是以所述储存稳定的药物组合物的水溶液形式提供用于注射或输注的药物制剂。

[0038] 通过在水性溶剂(例如注射用水(WFI))中重构步骤(v)的冻干粉末来获得药物制剂。

[0039] 药理学活性化合物的浓度范围可以为0.05mg/ml至40mg/ml。在一实施方式中，药理学活性化合物的浓度范围为0.1mg/ml至30mg/ml。更优选地，药理学活性化合物的浓度范围可以为0.5mg/ml至20mg/ml。更优选地，药理学活性化合物的浓度范围可以为0.75mg/ml至10mg/ml。所述药理学活性化合物的浓度可以最优选为约1mg/ml。

[0040] 制剂的pH在4以下。所述制剂的pH取决于药理学活性化合物的浓度，并且通常在0.5至4的范围内。例如，具有1mg/ml药理学活性化合物的制剂的pH在2至3的范围内。

[0041] 重构可以在一个或多个步骤中进行，例如，通过加入第一量溶剂溶解冻干物，随后将溶剂加至所需最终浓度。

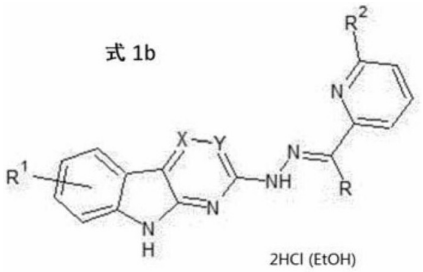
[0042] 用于重构含有所述药理学活性化合物的冻干粉末的水性溶剂还可以包含如上所述的药学上可接受的赋形剂。

[0043] 本发明的另一个方面是提供一种缓解、减轻或治疗对象中的癌症的方法，该方法通过单独使用本发明的药物组合物、或本发明药物组合物与另一抗癌治疗组合使用来进行。

[0044] 药物制剂的给药途径可以是通过输注或注射。然而，可以使用制剂或组合物给药的任意合适途径。制剂或组合物可以如下方式给药：例如，动脉内、肌肉内、胸膜内、口服、直肠、肠道、伤口内或肿瘤内和鞘内给药。

[0045] 本发明的另一个目标是提供通式1b示例的沉淀物，

[0046] 式 1b



[0047] 其中，至少95重量％(w/w)的通式1b的药理学活性化合物呈E-异构体的形式。

[0048] E-异构体的量的范围可以与上述药物组合物相同。

[0049] 通式1b的化合物是式1的吲哚衍生物的沉淀物，其中，取代基R、R^1、R^2、X和Y如式1中所限定。通式1b的优选化合物如表1所列。通式1b的最优选的化合物是如表1中的化合物A、B和C那样取代的。

[0050] 本发明的另一目的是提供用于制备含有如上所述的所述化合物或其药学上可接受盐的沉淀物的方法，所述方法对应于如上所述用于药物组合物的方法的步骤i)至iii)。

[0051] 一方面中，乙醇中的二盐酸盐(即，步骤ii)以两步骤添加，其中，1.0至1.15当量的乙醇中的盐酸在第一步骤中添加，并且2.0至2.5当量的乙醇中的盐酸在第二步骤中添加。或者，添加可以在一个或多个步骤中进行。盐沉淀物在步骤(ii)中自发溶解。

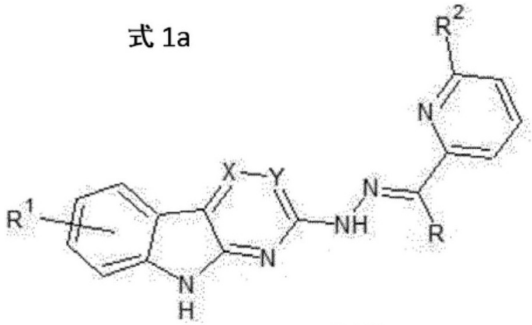
[0052] 如上所述沉淀物还可以在药物组合物中使用。

[0053] 沉淀物可以在进一步加工成冻干物之前直接使用或在干燥后使用。

[0054] 所述沉淀物的乙醇含量范围为所述沉淀物的2重量％至15重量％。优选在所述沉淀物的4重量％至13重量％、或9重量％至11重量％的范围内。在一实施方式中，乙醇的量为所述沉淀物的10.4重量％至10.6重量。

[0055] 本发明还提供含有通式1a的化合物的冻干物，

[0056] 式 1a



.2HCl

[0057] 其中，至少95重量％(w/w)的通式1a的药理学活性化合物呈E-异构体的形式。E-异构体的量的范围可以与上述药物组合物相同。

[0058] 通式1a的化合物是如上所述式I的吲哚衍生物的二盐酸盐。

[0059] 最优选的化合物是如上述式1和1b那样取代的。

[0060] 本发明还提供一种制备所述冻干物的方法，所述方法包括以下步骤：

[0061] a)将通式1b的沉淀物溶解于水性溶剂中，

[0062] b)所获得的溶液进行过滤，

[0063] c)将步骤b)的溶液进行冷冻干燥以获得含有通式1a的化合物的冻干物。

[0064] 在一方面中，沉淀物可以在搅拌下在步骤a)中溶解于水性溶剂中。所述方法在详细说明中进行进一步说明。

[0065] 步骤a)的沉淀物可以如式1或1b所述的任一种那样取代。在另一方面，沉淀物可以包含所述化合物中的一种或其组合。在另一方面，可以混合包含不同本发明化合物的单独的沉淀物。

[0066] 水性溶剂还可以包含至少一种药学上可接受的赋形剂。赋形剂和赋形剂的浓度可以如上所述。

[0067] 步骤b)所获得的溶液可以优选通过至少一个无菌过滤器进行过滤，在一些实施方式中，步骤b)所获得的溶液通过两个无菌过滤器进行过滤。所获得的溶液可以例如在步骤c)之前回收在无菌容积中。步骤b)的溶液还可以填充到适用于冷冻干燥的小瓶中。

[0068] 本发明的另一个目的是提供如上所述沉淀物或冻干物在药物组合物中的用途。

[0069] 本发明的药物组合物(即，冻干物)和沉淀物在室温下稳定至少12个月。优选地，药物组合物(即，冻干物)和沉淀物在室温下稳定至少24个月。

[0070] 本发明的另一个目的是提供组合物(即，含有所述化合物的冻干物)在治疗癌症中的用途。

[0071] 一方面中，本发明的冻干物可以仅包含一种本发明的药理学活性化合物，例如，化合物A2、B2、C2。在另一方面，本发明的冻干物可以包含本发明的化合物的组合。另一方面，本发明的含有所述化合物或药学上可接受的盐的冻干物可以包含至少一种本发明的化合物与至少一种用于癌症治疗的其它药理学活性化合物的组合。

[0072] 本发明的化合物可以单独给药或作为混合物给药。化合物还可以在另一药物或抗癌治疗的同时或之前或之后进行给药。

[0073] 上述药物组合物、沉淀物或制剂可以例如用于预防或治疗以病理性增殖细胞为特征的疾病或病症。

[0074] 药物制剂可以适用于通过在水性溶剂中使得所述组合物重构来进行输注或注射。优选地，制剂用于输注。

[0075] 药理学活性化合物的最终浓度范围可以为0.5mg/ml至30mg/ml。

[0076] 药物组合物和制剂的pH范围可以为0.5至4。优选地，pH范围为1至3。如上所述，pH取决于药理学活性化合物的浓度，并且例如，对于1mg/ml的制剂，pH范围为2至3。

[0077] 药物组合物或制剂还可以包含共治疗剂(co-therapeutic agent)。

[0078] 优选地，本发明的药物组合物和制剂用于治疗癌症。

[0079] 癌症可以是实体肿瘤、液体肿瘤或血液肿瘤。

[0080] 此外，上述药物、药物制剂、组合物、沉淀物或冻干物可以与另一抗癌治疗组合使用，例如，化疗、免疫或免疫调节疗法、激素疗法、肿瘤的手术切除、光动力疗法、激光疗法、热疗、冷冻疗法、血管生成抑制、放射疗法、或它们的组合。

[0081] 本发明还提供了用于治疗以病理性增殖细胞为特征的疾病或病症(如癌症)的方法，其中，将有效量的本发明的药理学活性化合物给予需要该治疗的对象。

[0082] 有效量的一种或多种所述所述药理学活性化合物在个体和癌症形式中是不同的。例如，有效量为约0.1-10mg/kg体重，优选约0.5-5mg/kg体重，并且更优选1-4mg/kg体重。给予对象的总剂量可以为5mg至800mg，取决于对象的状况和癌症形式，并且与所述对象的重量无关。一方面，给药至对象的剂量范围为30mg至300mg。当与如下所列举的另一癌症治疗组合时，该剂量甚至可以更低。

[0083] 在另一方面，本发明提供了如上所述癌症治疗方法与另一抗癌治疗组合的方法。

[0084] 如上所述的不同实施方式可以彼此组合使用或单独使用。

[0085] 下文的发明详述阐明了本发明的一个或多个实施方式的细节。本发明的其它特征、目的和优势通过描述和附图以及通过引用纳入本文的权利要求书将是显而易见的。

[0086] 附图的简要说明

[0087] 以下附图是对本发明的各方面的说明，并不意味着对权利要求所包含的本发明的范围进行限制。

[0088] 图1显示了用于合成化合物A的沉淀物(A1)的合成路径，以及沉淀物至对应盐(即，冻干物(A2))的盐形成步骤。

[0089] 图2a显示了99.8％纯度的化合物A1的HPLC色谱，图2b显示了通过X射线色谱确认的化合物A1的E-异构体结构。

[0090] 图3显示了化合物A在各种细胞系中的剂量-响应曲线。

[0091] 图4a-d显示了化合物A、B和C在HCT116-细胞中剂量-响应曲线(A)，以及化合物A(b)、化合物B(c)和化合物C(d)在HepG2-细胞、RKO-细胞、HeLa-细胞、CEM-细胞和THP-1细胞中的剂量-响应曲线。

[0092] 发明详述

[0093] 应该理解，本发明不限于本文所公开的具体配置、方法步骤和材料，因为这些配置、方法步骤和材料可以在一定程度上变化。还应理解，本文所用术语的目的仅是描述具体实施方式，不用来构成限制，因为本发明的范围仅受所附权利要求书及其等价形式的限制。

[0094] 本文引用的所有参考文献通过引用全文纳入本文并用于所有目的，就好像将各篇单独的出版物、专利或专利申请特定和单独地通过引用全文纳入本文用于所有目的一样。

[0095] 参考本文提供的以下定义,附图和示例性公开,可以最好地理解本发明。

[0096] 在本说明书中,通式I的化合物意在包括其任何药学上合适的沉淀物、溶剂合物、盐或前药。

[0097] 在本说明书中,术语“沉淀物”表示通过沉淀得到的二盐酸盐乙醇共结晶化合物、或二盐酸盐乙醇盐、或二盐酸盐乙醇溶剂合物,例如在图1的反应4中沉淀步骤的产物。化合物可以是本发明的任意式1的化合物的沉淀物。

[0098] 在本说明书中,术语“药学上可接受的化合物”包括本说明书中所述的沉淀物、溶剂合物和冻干物。

[0099] 在本说明书中,术语"异构体"是指具有相同组成和分子量但物理和/或化学性质不同的化合物。这种物质具有相同的原子数量和种类,但是结构上不同。结构差异可以是在构造上(几何异构体)或在偏振光平面的旋转能力上(立体异构体)。术语“立体异构体”是指原子空间排列不同的相同构造的异构体。

[0100] 在本说明书中,除非另有说明,否则术语“药学上可接受的赋形剂”是指无毒、惰性的固体、半固体或液体填料、稀释剂、包封材料或任何类型的制剂助剂。

[0101] 在本说明书中,除非另有说明,否则术语“药理学活性化合物”包括当给药于宿主(包括人类和动物)时将产生治疗有益的药理学响应的任何物质。

[0102] 在本说明书中,术语“给予”或“给药”是指以药学上可用方式将药物提供给对象。

[0103] 在本说明书中,除非另有说明,否则术语“细胞毒性化合物”是指能够阻止细胞生长或杀死细胞(即具有高细胞毒活性)的化合物。

[0104] 在本说明书中,除非另有说明,否则术语“衍生物”是指由初始结构直接形成的化合物、或通过初始结构的化学反应形成的化合物、或通过“修饰”(其是初始结构的部分取代)形成的化合物、或通过设计和从同合成形成的化合物。衍生物可以是合成的,或者可以是细胞或体外酶促反应的代谢产物。

[0105] 在本说明书书中,术语“癌症”是指任何恶性肿瘤疾病,即由异常和不受控制的细胞分裂引起的任何恶性生长或肿瘤。术语“癌症”具体意味着包括实体局部肿瘤和非实体癌症形式。例如,所述癌症形式可以选自:白血病(ALL、AML、CLL、CML、CMML)、T细胞白血病、多发性骨髓瘤、卵巢癌、前列腺癌、宫颈腺癌、鳞状细胞癌、乳腺癌、结直肠癌、小肠癌、肛门癌、胃癌、肾癌、肾盂和输尿管的恶性黑色素瘤、尿道癌、膀胱癌、肝癌、阑尾癌、胰腺癌、肺癌、食道癌、唇癌/口腔癌、鼻癌、喉癌、脑/中枢神经系统癌症、皮肤癌、甲状腺和胸腺癌、肉瘤、头颈癌、非霍奇金淋巴瘤(NHL)、霍奇金淋巴瘤和腹膜假粘液瘤。

[0106] 本发明提供了用于制备有利于E-异构体的药物组合物的方法。单晶X射线证实E-异构体在固态下占主导地位。

[0107] 通过使用本发明的方法,获得了包含至少95重量%(通过HPLC确认,参见图2)的药理学活性化合物(E-异构体)的定义明确且稳定的药物组合物。

实施例

[0108] 实施例1:化合物A的合成

[0109] 在第一个实验中,化合物A(游离碱)在丙酮/乙酰化物/乙腈(acetone nitrile)中稀释,E-异构体可溶于该溶剂组合中但Z-异构体不能,并且易于过滤。使用该溶剂组合的最

终E-异构体含量约为92％。所述溶剂组合在小规模生产过程中运行良好，但由于需要大量溶剂而无法扩大生产。因此，发明人基于由Kgokong等人2005年所描述的1,2,4-三嗪并[5,6-b]吲哚衍生物合成研发了化合物A的合成(参见图1)。发明人研发了使用甲醇(MeOH)作为溶剂、以及乙醇(作为HCl的载体)中的盐酸(HCl/EtOH)(EtOH也用作反溶剂(anti-solvent))。在随后的放大工艺研发中，反应体积效率得到改进。此外，还研发了用于大规模将游离碱(A)转化为盐酸盐沉淀物(A1)的合适方法(参见图1，实施例1和2)。游离碱(A)不溶于单独的MeOH，但是在加入约1当量的HCl/EtOH时获得了清澈的溶液。

[0110] 由于观察到的二硫化物物质，反应可以在氮气下进行以避免空气氧化。由反应步骤1产生的湿滤饼也可以在真空中干燥，或者可以在没有事先干燥的情况下进一步处理湿滤饼。通过真空干燥使得杂质的产生最小化，因为杂质可能在抽气干燥过程中产生。在50℃下使反应步骤2的产物化合物与略过量的2-乙酰基吡啶(1.5当量)在乙醇(20mL/克化合物)中反应以形成产物，但在5小时后转化率过低(～8％)。

[0111] 图1显示了化合物A的合成的反应步骤1-3(E和Z异构体的混合物；IUPAC系统名称：2-[(1E)-1-(2-{6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚-3-基}肼-1-亚基)乙基]吡啶)。

[0112] 步骤1.向7-甲基靛红(methylisatin)(4.75kg，29.5mol)的水性悬浮液中加入2.85kg(31.3mol)氨基硫脲和6.15kg(44.5mol)碳酸钾。将经搅拌的混合物在回流下加热3小时，然后冷却至室温。缓慢加入乙酸(100％，3.3kg，55.0mol)，直至pH达到7.1。用压滤机过滤悬浮液，滤饼用水(19.4kg)洗涤以获得7.6kg湿的6-甲基-2H,3H,5H-[1,2,4]三嗪并[5,6-b]吲哚-3-硫酮。

[0113] 步骤2.将来自前一步的对应于约4.6kg干燥的6-甲基-2H,3H,5H-[1,2,4]三嗪并[5,6-b]吲哚-3-硫酮的湿滤饼悬浮于57.1kg的一水合肼，并将混合物在89℃下搅拌18小时。使反应混合物冷却到室温，产物通过离心进行分离，用水(15.9kg)和乙醇(18.4kg)洗涤，在1450RPM下排水。将3-肼基-6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚的湿滤饼(7.8kg相当于3.8kg干重)转移回清洁的反应器中并在真空下干燥。

[0114] 步骤3.向来自步骤2的干燥的3-肼基-6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚中加入水(76.85kg)、乙酸(100％，6.70kg，111.6mol)和2-乙酰基吡啶(10.75kg，88.7mol)。将混合物在48.5℃搅拌3小时，冷却至室温，并在将温度保持在20-25℃之间的同时缓慢加入NaOH(27％，6.3kg，110mol)以达到pH7.0。在此温度下将混合物进一步搅拌$1^{1}/_{4}$小时，并通过离心分离产物。在用水(7.3kg)和乙醇(5.8kg)的混合物洗涤后，滤饼在1450RPM下排水，然后在47℃的真空烘箱中干燥66小时，得到5.82kg形式为米色/带绿色的固体材料的标题化合物。

[0115] 图1中的步骤4显示了化合物A1的合成，其是化合物A的乙醇共结晶(IUPAC系统名称：2-[(1E)-1-(2-{6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚-3-基}肼-1-亚基)乙基]吡啶二盐酸盐)。

[0116] 向2-[(1E,Z)-1-(2-{6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚-3-基}肼-1-亚基)乙基]-吡啶)(5.80kg)中加入乙醇HCl(12.4kg，1.05当量)，将混合物在28-30℃下搅拌半小时直至获得澄清溶液。溶液进行过滤并在搅拌下在25℃下在1小时40分钟内加入另外的乙醇HCl(28.95kg，2.45当量)。在第一次添加1.05当量HCl/EtOH的过程中，大部分Z-异构体转化为E-异构体并形成一些单盐酸盐。通过加入2.45当量的EtOH中的HCl，二盐酸盐自发沉

淀。通过用0.1M NaOH酚酞指示剂滴定EtOH中HCl的摩尔浓度，计算出约1.1至1.4M HCl。在相同温度下继续搅拌15分钟并加入乙醇(45.8kg)。将如此形成的悬浮液冷却至约0至-5℃并搅拌1小时。离心分离的产物用乙醇(0至5℃，45kg)洗涤，然后在1450RPM下排水。将滤饼在37℃下真空干燥42小时，以得到7.57kg标题化合物(基于无残留溶剂-游离碱为约108％或基于单EtOH为98％，二盐酸盐为黄色至橙色固体)。

[0117] 所获得的乙醇共晶二盐酸盐沉淀物具有约2重量％至20重量％的乙醇含量。

[0118] 图1中的反应步骤5显示了包含通式1a的化合物的冷冻干燥组合物的形成。

[0119] 通过HPLC分析异构体含量

[0120] 在方法研发过程中，化合物A和化合物A1的分析导致了分析问题，例如由于如样品不稳定、溶解度差、异构化、HPLC等。因此，发明人基于XBridgeC18,3.5μm,150×4.6mm柱研发了更稳健的HPLC方法。通过使用MeOH中的2％甲酸作为稀释剂，并从未涂覆的标准HPLC样品瓶转换为来自安捷伦(Agilent)的经涂覆的(硅烷化)小瓶，进一步解决了该问题。

[0121] 使用Agilent 1200/1260色谱系统或等同物。

[0122] 当使用酸性HPLC分析化合物A时，发现～7％为Z-异构体形式(在0.1％TFA/H_2O中制备样品)。2天后重新分析同样的样品，显示出～2％的Z-异构体，并开始水解成化合物A1(检测到约1％)。这表明酸性条件(pH范围为1-4)使得不期望的Z-异构体转化为所需的E-异构体。当进行随后的盐形成(反应步骤4)(使用乙醇中的HCl)时，异构体含量降低至<0.5％。这意味着化合物A、B或C可以允许相对较大含量的不期望的异构体(例如5％)，因为在加入乙醇中的HCl后不期望的异构体将转化成所需的异构体。乙醇中的HCl的加入形成了二盐酸盐沉淀物(如化合物A1、B1和C1)。

[0123] HPLC纯度

[0124] 将HPLC纯度计算为100％-杂质总量。计算中排除所有低于0.05％的峰和基质中存在的峰。各杂质的含量计算为总峰面积的百分比(面积％)。杂质总量是≥0.05％的杂质总和。

[0125] 杂质

[0126] 各杂质的最终结果是四个结果的平均值。杂质总量记录为≥0.05％的杂质总和。

[0127] 残留溶剂

[0128] 化合物A1的分析表明它是二盐酸盐乙醇共结晶组合物(沉淀物)。化合物A1的理论乙醇含量为10.6％，这与如上所述的乙醇共结晶(沉淀物)的形成一致。

[0129] 在包含化合物A的组合物的工艺研发期间，令人惊讶地显示二盐酸盐乙醇共结晶(例如A1)吸湿性较小并且对水解和异构体纯度的降解显著更稳定。

[0130] 得出的结论是，药物物质(沉淀物)中可以容忍高水平的乙醇，因为它在随后的冷冻干燥期间被除去，后续冷冻干燥是最终药物产品(冻干物)的制造工艺的一部分。

[0131] 甲醇水平显示相对较高；通常组合物A1的甲醇水平为1.4-1.8％。长时间的干燥循环不会显著降低甲醇水平。然而，如用乙醇的情况下，在最终药物产物(例如A2)的制造期间所使用的随后的冷冻干燥循环有效地将甲醇去除至低于ICH Q3B指南的水平。

[0132] 结论

[0133] 基于最终药物产物中乙醇和甲醇水平远低于ICH Q3C指南的事实，并且考虑到这一点被仔细监测，得出的结论是药物物质(即，化合物A1)中可允许较高水平。说明书中说明

的所有其他限制均在Ph.Eur或USP标准范围内。

[0134] 鉴定

[0135] 样品的鉴定是基于样品制备的主峰和用于鉴定的样品制备的主峰的目视检查。化合物A1由色谱图中的一个单峰表示(参见图2a)。

[0136] 实施例2:稳定性

[0137] 根据国际药品注册协调会议(International Conference on Harmonization,ICH)指南Q1A(R2)新药物物质和产品的稳定性测试,进行二盐酸盐乙醇共结晶沉淀物和冻干二盐酸盐的稳定性研究。在研究期间用于分析稳定性样品的所有分析仪器均符合当前cGMP的要求。

[0138] 稳定性研究由两部分组成,一个长期研究(5℃,24、36个月)和一个加速研究(25℃/60%RH,6个月)。

[0139] 将化合物A(A1)的二盐酸盐乙醇共结晶沉淀物装入置于密闭HDPE容器中的热封箔片层叠袋内的热封双层聚乙烯袋中。样品在长期条件5℃和加速条件25℃/60%RH下储存。整个测试期间外观呈黄色至橙色固体。由于25℃/60%RH的X射线粉末衍射结果而进行的分析具有意想不到的低结晶度水平。结晶度水平对药物物质的质量或稳定性没有直接影响,但作为研发工作的一部分进行控制。所获得的36个月的稳定性数据总结在下表2a中。

[0140] 表2b显示了在6个月的时间内25℃和60%RH下二盐酸盐乙醇共结晶沉淀物的稳定性数据。整个过程中外观呈黄色至橙色固体。

[0141] 结论

[0142] 包含化合物A1的本发明组合物稳定至少24个月(表2a)。在此期间,在2-8℃或25℃/60%RH(6个月)下化合物A1没有出现显著分解。其表示化合物A1的组合物应在2-8℃储存和运输。但是,在最高达25℃的温度下储存24小时应该没有问题。

[0143] 实施例3:2-[(1E)-1-(2-{6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚-3-基}肼-1-亚基)乙基]-吡啶二盐酸盐的乙醇共结晶沉淀物的药物组合物的制备

[0144] 将多个225.6mg的主要2-[(1E)-1-(2-{6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚-3-基}肼-1-亚基)乙基]-吡啶二盐酸盐的乙醇共结晶沉淀物(A1)(相当于160mg游离碱,A)溶于甘露醇(500mg)的注射用水溶液中(Ph.Eur.,10ml)中,溶液通过两个0.2μm过滤器过滤来灭菌并填充到相应数量的经灭菌的小瓶中,然后冷冻干燥(获得化合物的盐A2)。

[0145]

表 2a

时间（月）		0	1	3	6	9	12	18	24	36
RRT 0.92-0.93	≤1.0	0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.07	<0.05	0-07
RRT 1.13	≤1.0	<0.05	<0.05	<0.06	<0.06	<0.05	0.05	0.05	0.05	<0.05
RRT 1.23-1.24	≤1.0	0.05	0.05	0.06	0.06	0.05	0.06	0.06	0.06	0.06
	≤1.0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.05	<0.05	<0.05

[0146]

RRT 1.39	≤1.0	0.17	0.10	0.10	0.36[2]	0.09	<0.05	0.06	0.05	0.06
RRT 1.47-1.51	≤2.0	0.26	0.15	0.16	0.42	0.14	0.10	0.29	0.16	0.18
总杂质										
含水量(% w/w)		2.53	2.34	2.18	2.50	2.88	2.65	2.73	3.37	2.46

[0147] [2]RRT＝1.47-1.51时杂质的相对面积高于预期。由另一位分析师重复了样品制备和HPLC分析，证实了结果。在测试方法验证期间观察到这种杂质的波动峰面积。

[0148] 发明人研发了新的冷冻干燥方法，因为现有技术使用的常规方法需要超过300小时的干燥。新方法更具好，并列于下表3中。

[0149]

表 2b

时间（月）		**0**	**1**	**3**	**6**
RRT 0.92-0.93	≤ 1.0	0.05	< 0.05	0.05	0.07
RRT 1.12	≤ 1.0	< 0.05	< 0.05	< 0.05	0.05
RRT 1.24	≤ 1.0	0.05	0.05	0.06	0.05
RRT 1.38	≤ 1.0	< 0.05	< 0.05	0.05	< 0.05
RRT 1.49-1.51	≤ 1.0	0.17	0.10	0.06	0.36[2]
总杂质	≤ 2.0	0.26	0.16	0.22	0.52
含水量 (% w/w)		2.53	2.41	2.30	2.45

[0150] [2]RRT＝1.47-1.51时杂质的相对面积高于预期。

[0151] 由另一位分析师重复了样品制备和HPLC分析，证实了结果。在测试方法验证期间观察到这种杂质的波动峰面积。

[0152] 通过具有如表3所示的最大负压和相对高的温度、退火温度，冷冻干燥步骤减少到19小时。

[0153] 避免与金属表面接触。通过冷冻干燥过程除去存在的乙醇和少量甲醇。

[0154] 小瓶在氮气下钳口密封并储存在5℃下；储存24个月后没有观察到降解。

[0155] 评价作为赋形剂的葡萄糖和甘露醇单独使用、或与NaCl组合使用。用5％(w/v)甘露醇作为添加剂获得了关于溶解度、冻干饼的质地和抑制杂质形成的最佳结果。用葡萄糖作为膨胀剂(bulking agent)观察到经冷冻干燥的饼更高程度的塌陷。由于NaCl所产生的pH增加降低了化合物A2的溶解度，所以添加NaCl会引起溶解度问题。

[0156] 用于重构和注射的冻干粉末(相当于160mg游离碱化合物A)在2-8℃条件下储存至

24个月。整个试验期间外观呈黄色至橙色的经冷冻干燥的饼，并在重构后是黄色至橙色溶液而没有可见颗粒。

[0157] 表3

步骤类型	温度（T°C）	时间(小时)	真空（mbar）
货架（shelves）	5	/	/
冷冻步骤	5	0.30	/
冷冻跃迁（Freezing ramp）	-45	0.50	/
冷冻步骤	-45	4	/
冷冻跃迁（退火）	-25	1	/
冷冻步骤（退火）	-25	2	/
冷冻跃迁（退火）	-45	1	/
冷冻步骤	-45	4	/
腔室真空	-45	/	0.200
初步干燥	-45	0.10	0.200
初步干燥跃迁（drying ramp）	25	3	0.200
初步干燥步骤	25	XX*	0.200
第二干燥跃迁（drying ramp）	25	10	最大
循环终止			

[0158]

[0159] 由于25℃/60％RH的X射线粉末衍射结果而进行的分析具有意想不到的低结晶度水平。结晶度水平对药物物质的质量或稳定性没有直接影响，但作为研发工作的一部分进行控制。重构时间最长达3分钟。没有检测到细菌生长，并且在室温下的24个月期间不影响产品的无菌性。获得的稳定性数据总结在下表4a中。

[0160]

表 4a

时间（月）	0	1	3	6	12	18	24
pH	1.6	1.6	1.6	1.5	1.6	1.6	1.7
含水量(%)	0.33	0.41	0.47	0.37	0.53	0.43	0.39
分析(% w/w)[1]	97.8	98.4	95.9	97.3	93.9	94.6	94.2
总杂质(%)	0.7	0.5	0.3	0.4	0.2	0.3	0.34
任意个体纯度(%)	0.4	0.3	0.1	0.1	0.1	0.1	0.10
特定杂质*(%)	<0.5	<0.05	<0.05	0.08	0.08	0.10	0.09
Z-异构体 (%)	0.1	0.1	0.1	0.1	<0.05	<0.05	0.05
RRT 0.92-0.93							
RRT 1.13	0.05	<0.05	<0.05	<0.05	<0.05	0.05	<LOQ
RRT 1.23-1.24	0.06	0.05	0.08	0.05	0.06	0.05	0.05
RRT 1.39							
RRT 1.47-1.51	0.37	0.32	0.05	<0.05	<0.05	0.05	0.10

[0161] *水解杂质3-肼基-6-甲基-5H-[1,2,4]三嗪醇[5,6-b]吲哚

[0162] LOQ为0.05％，<LOQ的峰记录<0.05％。

[0163] 用于重构和注射的冻干粉末(相当于160mg游离碱化合物A)在25℃/60％RH加速条件下储存(参见表4b)。整个试验期间外观呈黄色至橙色的经冷冻干燥的饼，并在重构后是黄色至橙色溶液而没有可见颗粒。由于25℃/60％RH的X射线粉末衍射结果而进行的分析具有意想不到的低结晶度水平。结晶度水平对药物物质的质量或稳定性没有直接影响，但作为研发工作的一部分进行控制。重构时间最长达3分钟没有检测到细菌生长，并且在室温下的24个月期间不影响产品的无菌性。令人惊讶的是，冻干物在室温下显示出稳定至少24个月。获得的稳定性数据总结在下表4b中。

[0164]

表 4b

时间（月）	0	1	3	6	12	18	24
pH	1.6	1.6	1.6	1.5	1.6	1.5	1.6
含水量(%)	0.33	0.39	0.55	0.45	0.59	0.49	0.45
分析(% w/w)[1]	97.8	97.2	95.4	98.3	94.6	93.9	94.5
总杂质(%)	0.7	0.3	0.7	0.4	0.2	0.20	0.36
任意个体纯度(%)	0.4	0.1	0.2	0.1	0.1	0.1	0.10
特定杂质*(%)	<0.5	0.1	<0.05	0.08	0.08	0.10	0.10
Z-异构体 (%)	0.1	<0.05	0.1	0.1	0.10	<0.05	0.05
RRT 0.92-0.93 RRT 1.13	0.05	<0.05	<0.05	<0.05	<0.05	nd	<LOQ
RRT 1.23-1.24 RRT 1.39	0.06	0.05	0.08	0.06	0.06	0.06	0.06
RRT 1.47-1.51	0.37	0.13	0.05	<0.05	<0.05	nd	0.09

[0165] *水解杂质3-肼基-6-甲基-5H-[1,2,4]三嗪醇[5,6-b]吲哚

[0166] LOQ为0.05％，<LOQ的峰记录<0.05％。pH应该在0.5-4的范围内，在上面的实施例中，浓度约为16mg/ml，pH值在1.3-2.3范围内，并且含水量在1％以下。Z-异构体优选小于2％，然而发明人惊讶地发现酸性条件有利于E-异构体。

[0167] 包含化合物A2的冻干物令人惊讶地显示出在冷冻干燥之后比在冷冻干燥之前水溶性更小。由此，进行了结构调查，并且该研究显示出化合物A2在冷冻干燥期间会改变其结晶形式。新的结晶形式在水中难溶，这解释了二盐酸盐乙醇共结晶沉淀(A1)和二盐酸盐(A2)之间的溶解度差异。实验表明，该沉淀物的耗尽引起形态形式的变化。实验结果还显示，赋形剂(D-甘露糖醇)对新形态形式的形成没有任何影响。用表3中所述的冷冻干燥循环和设定为5％的甘露醇含量获得了关于杂质形成和冻干饼质地的最佳结果。

[0168] 实施例4:药物制剂的制备

[0169] 发现化合物A2可以以1mg/ml配制在水性介质中以抑制副产物的形成最长达24小

时。而且，应理解，pH对于化合物A2在水性介质中的稳定性具有重要意义，在约1-4的pH下具有最好的稳定性，物质的浓度越高导致的pH越低。1mg/ml的所述水溶液具有约2-3的pH。

[0170] 将得到的化合物A2配制为无菌冻干粉末，将上述冻干粉末溶解于水性溶剂(如注射用水)中来制备注射用或输注用溶液。各小瓶含有一定量的药理活性化合物，对应于由225.6mg药物(A1)和5％甘露醇(w/v)的溶液制备的160mg游离碱(A)。冻干物可以在10ml水性溶剂中重构，然后在任选地包含药理学上可接受的赋形剂、优选5％甘露醇(w/v)的水性溶剂中稀释至1mg/ml，用于输注。

[0171] 实施例5

[0172] 化合物B的合成；2-[(1E)1-(2-{6-甲基-5H-[1,2,4]三嗪并[5.6-b]吲哚-3-基}肼-1-亚基)丙基]吡啶

[0173] 将1-(吡啶-2-基)丙-1-酮(35mg，0.26mmol)溶于水-乙酸混合物(20:1,10mL)中，然后加入3-肼基-6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚(50mg，0.23mmol)。将反应混合物在50℃下搅拌2小时。在蒸发溶剂后，得到深绿色固体(70mg)。LC显示出异构体比例为95:5的纯产物。

[0174] 实施例6

[0175] 化合物C的合成；2-(3,3-二甲基-N-{6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚-3-基}丁烷腙基)吡啶

[0176] 将3,3-二甲基-1-(吡啶-2-基)丁-1-酮(46mg，0.26mmol)溶于水-乙酸混合物(20:1,10mL)中，然后加入3-肼基-6-甲基-5H-[1,2,4]三嗪并[5,6-b]吲哚(48mg，0.23mmol)。反应混合物在50℃下搅拌过夜。在蒸发溶剂后，得到黄绿色(greenish yellow)固体(78mg)。LC显示出异构体比例为92:8的纯产物。

[0177] 实施例7

[0178] 化合物B1向其二盐酸盐(B2)的转化通过以下程序制备：

[0179] 将2-[(1E)1-(2-{6-甲基-5H-[1,2,4]三嗪并[5.6-b]吲哚-3-基}肼-1-亚基)丙基]吡啶(30mg，0.09mmol)悬浮在甲醇(0.6mL)中，然后逐滴加入乙醇中的HCl(1.04当量，1.28M，75μL)。在所有固体溶解后，加入更多的乙醇中的HCl(2.08当量，1.25M，150μL)和乙醇(0.6mL)。出现浅棕色沉淀。将悬浮液在-10℃保持3小时，然后过滤固体，用冷乙醇洗涤并干燥。产物是亮黄色固体(10mg)。LC仅显示一种异构体，在将产物转化成其HCl盐后未检测到次要异构体。

[0180] 实施例8

[0181] 化合物C1向其二盐酸盐(C2)的转化通过以下程序制备：将2-[(1E)1-(2-{6-甲基-5H-[1,2,4]三嗪并[5.6-b]吲哚-3-基}肼-1-亚基)丙基]吡啶(30mg，0.09mmol)悬浮在甲醇(0.6mL)中，然后逐滴加入乙醇中的HCl(1.04当量，1.28M，75μL)。在所有固体溶解后，加入更多的乙醇中的HCl(2.08当量，1.25M，150μL)和乙醇(0.6mL)。产物不会立即沉淀，仅在悬浮液在-10℃下保持3小时后。过滤进行固体，用冷乙醇洗涤并干燥。产物是亮黄色固体(20mg)。LC仅显示一种异构体(E)，在将产物转化成其HCl盐后未检测到次要异构体(Z)。

[0182] 表征

[0183] 单晶X射线显示出E-异构体在固态下占主导地位。

[0184] 单晶X射线在瑞典的SARomics Biostructures AB进行。在通常用于蛋白质晶体类

型的标准低温回路中，浸入石蜡油中并在液氮中闪蒸冷却，获得约100×30μm的化合物A1的晶体。在装有225mm mar CCD检测器的MAX-实验室(MAX-lab)的I911-3站在100K下收集数据（λ= 0.9198Å）。光束尺寸为50x 50μm。X射线结果证实化合物A1是E-腙异构体。预测结构如图2b所示，其中N代表氮原子，H代表氢原子，CL代表氯原子，H_2O代表水分子。

[0185] 所有测试均使用参考标准进行，并且所有分析均与所提出的结构相符。

[0186] 结论

[0187] 即使产物化合物A2容易在水性溶剂中水解，化合物A的原料中的4-7％的含水量也是可容许的。

[0188] 母液中没有异构体痕迹，表明所用的沉淀条件将Z-异构体转化为了目标E-异构体。优选地，盐形成应该在数小时内进行，因为产物对酸敏感。

[0189] 组合物研发工作从上文讨论的水性溶剂稳定性测试和赋形剂评估开始。基于这些结果，关于对杂质形成和药物产品(即，二盐酸盐，例如化合物A2)溶解度的影响，通过优化组成(即，药物物质-乙醇共结晶沉淀物-浓度和赋形剂的类型和数量)继续进行组合物研究。作为优化的结果，各小瓶游离碱(化合物A)的量从100增加到160mg。

[0190] 实施例9细胞毒活性

[0191] 在不同细胞系(图3)和人类肿瘤的原代培养物中显示化合物A的表示为存活指数(IC50)的细胞毒活性(表5)。微培养细胞毒性荧光测定法(FMCA)(Lindhagen等人，2008)用于测定化合物在各种细胞系和人类肿瘤原代培养物中的细胞毒性作用。使用移液机械手Precision 2000(伯腾仪器公司，佛蒙特州威努斯基(Bio-Tek Instruments Inc.，Winooski，VT))将细胞接种在药物制备的384孔板中。将板温育72小时，然后转移到由如下组成的整合HTS SAGIAN核心系统：具有CO_2孵育器(Cytomat 2C，Kendro，瑞典绍伦吐纳)的ORCA机器人(贝克曼库尔特公司(Beckman Coulter))、分配器模块(Multidrop 384，Titertek，亨兹维尔瑞典)、洗涤器模块(ELx405，伯腾仪器公司)、脱盖站、板收纳处(plate hotels)、条形码阅读器(贝克曼库尔特公司)、液体处理器(Biomek 2000，贝克曼库尔特公司)和用于自动化FMCA的多用途阅读器(FLUOstar Optima，BMG Labtech，德国奥芬堡)。

[0192] 对不同的细胞系(例如，CCRF-CEM T细胞白血病、RPMI-8226多发性骨髓瘤、A2780卵巢癌、FaDu头颈癌(鳞状细胞癌肿瘤)、HT29结直肠癌、MCF7乳腺癌和HL-60白血病细胞)以及原代人类肿瘤细胞培养物组(表5)进行分析(结肠癌、胃癌、肾癌、阑尾癌、小肠癌和胰腺癌、以及腹膜假性粘液瘤)。结果显示化合物A具有广泛的抗癌活性，如效果-浓度图(图3)所例示。

[0193] 实施例10

[0194] 本发明人还着手表征代表化合物A、B和C在不同来源的癌的细胞系中的活性。之前已经详细描述了所使用的特定分析和机理评估的结论(Zhang等人，2014)。如先前详细描述的(Lindhagen等人，2008)，使用基于细胞的微培养细胞毒性荧光测定(FMCA)评估化合物A、B和C(参见图4)的细胞毒性，其表达为六种人肿瘤细胞系中的存活指数。该方法基于测定由具有完整质膜的活细胞的FDA水解产生的发荧光的荧光素。荧光与完整活细胞的数量成比例。

[0195] 材料和方法

[0196] 细胞培养

[0197] 细胞系在供应商推荐的相应细胞培养基中进行培养。培养基补充有10％热灭活的胎牛血清、2mmol/L的L-谷氨酰胺、100μg/mL的链霉素和100U/mL的青霉素(均来自西格玛-奥德里奇公司(Sigma-Aldrich))。所有细胞系在含5％CO_2的潮湿气氛中于37℃进行培养。

[0198] 细胞毒活性的检测

[0199] 简要介绍FMCA分析，将每孔2500个细胞接种到384孔的微孔板中并在用化合物处理前温育过夜。使用声学液体传递(acoustic liquid transfer)(Echo 550,LabCyte)加入化合物。将板在37℃孵育72小时，然后洗涤并将FDA加入到孔中，然后在37℃孵育50分钟。在Fluoroskan仪器(Labsystems,GMI,Ramsey,MIN)中在485/520nm下测定荧光，所述荧光与各孔中的活细胞数量成比例。细胞存活表示为生存指数(SI)，定义为化合物处理过的孔中的荧光值，以对照孔中的值的百分比进行分析，扣除空白值。质量标准包括信号/空白比>10，对照和空白孔的变异系数(CV)<30％。Graph Pad Prism(美国加利福尼亚圣地亚哥)。所有实验进行两次，各浓度在各实验中以一式四份进行评估。化合物(A、B和C)用DMSO，5mM稀释。

表5.不同原代人类肿瘤细胞培养物组的IC_{50}

[0200]

疾病	进行分析的患者数：	IC_{50}μM
PMP*	50	9.4
结直肠肿瘤**	25	11
胃癌	9	6.9
肾癌	13	164
间皮瘤	7	12
阑尾癌	4	21
小肠癌	1	5.4
软巢癌	30	5.7
胰腺癌	1	6.0

[0201] *腹膜假粘液瘤，**大肠癌，从最大肿瘤细胞减灭术和腹膜切除术(peritinectomies)获得的手术标本

[0202] 结果和讨论

[0203] 所测试的化合物(A、B和C)在广泛的癌细胞系中显示出强烈的活性，参见表6和图4。选择细胞系以覆盖范围广泛的代表血液肿瘤和实体肿瘤两者的癌症类型(表6)。

[0204] 从这些结果清楚地显示出化合物A、B和C对几种不同的肿瘤细胞系是有效的，包括结肠癌、宫颈腺癌、肝细胞癌、急性淋巴母细胞性白血病和急性单核细胞白血病。

[0205] 从此处给出的结果清楚地显示出化合物A、B和C对几种不同的肿瘤细胞系是有效的，包括结肠癌、宫颈腺癌、肝细胞癌、急性淋巴母细胞性白血病和急性单核细胞白血病。

[0206] 表6.在六种人肿瘤细胞系中化合物A、B和C的IC_{50}

[0207]

细胞系	来源	IC_{50} 化合物A	IC_{50} 化合物B	IC_{50} 化合物C
HCT116	结肠癌	≈ 1 μM	≈ 1 μM	≈ 1 μM
RKO	结肠癌	< 250 nM	< 250 nM	< 250 nM
HeLa	宫颈腺癌	≈ 20 μM	≈ 20 μM	≈ 10 μM
HepG2	肝细胞癌	< 250 nM	< 250 nM	< 250 nM
CCRF-CEM	急性淋巴细胞白血病	< 250 nM	< 250 nM	< 250 nM
THP-1	急性单核细胞白血病	< 250 nM	< 250 nM	< 250 nM

[0208] 虽然已在本文中详细讨论了特定实施方式，但是这仅仅是为了说明的目的而通过示例的方式进行，并且不旨在限制所附权利要求的范围。具体而言，发明人考虑，可对本发明进行多种取代、变化和修改而不背离权利要求所限定的本发明的精神和范围。

[0209] ---

[0210] 参考文献

[0211] Eshba等人，作为潜在抗病毒剂和抗癌剂的一些经取代的-1,2,4-三嗪并[5,6-b]吲哚衍生物的合成(Synthesis of some substituted-1,2,4-triazino[5,6-b]indole derivatives as potential antiviral and anticancer agents)，药学(Pharmazie)第42卷，第10期，1987;664-666。

[0212] Lindhagen E,Nygren P,Larsson R(2008)，微培养细胞毒性荧光测定(The fluorometric microculture cytotoxicity assay)，Nature Protocls 3:1364-1369。

[0213] Kgokong JL,Smith PP,Matsabisa GM(2005)Bioorg Med Chem.13(8):2935-42)。

[0214] Zhang X等人(2014)，在代谢受损的微环境中诱导线粒体功能障碍作为靶向肿瘤细胞的策略(Induction of mitochondrial dysfunction as a strategy for targeting tumor cells in metabolically compromised microenvironments)，Nature communications 5:3295。

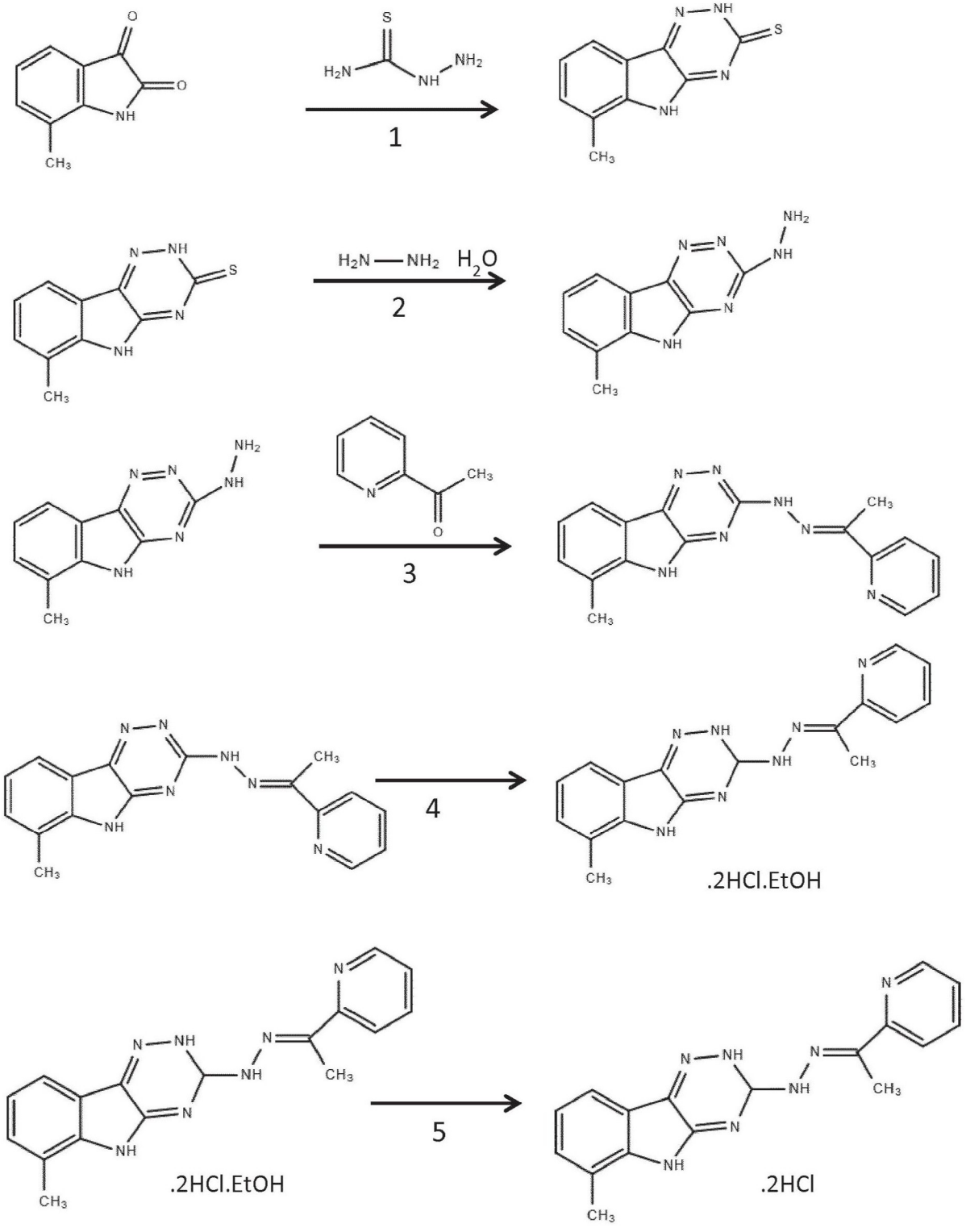


图1

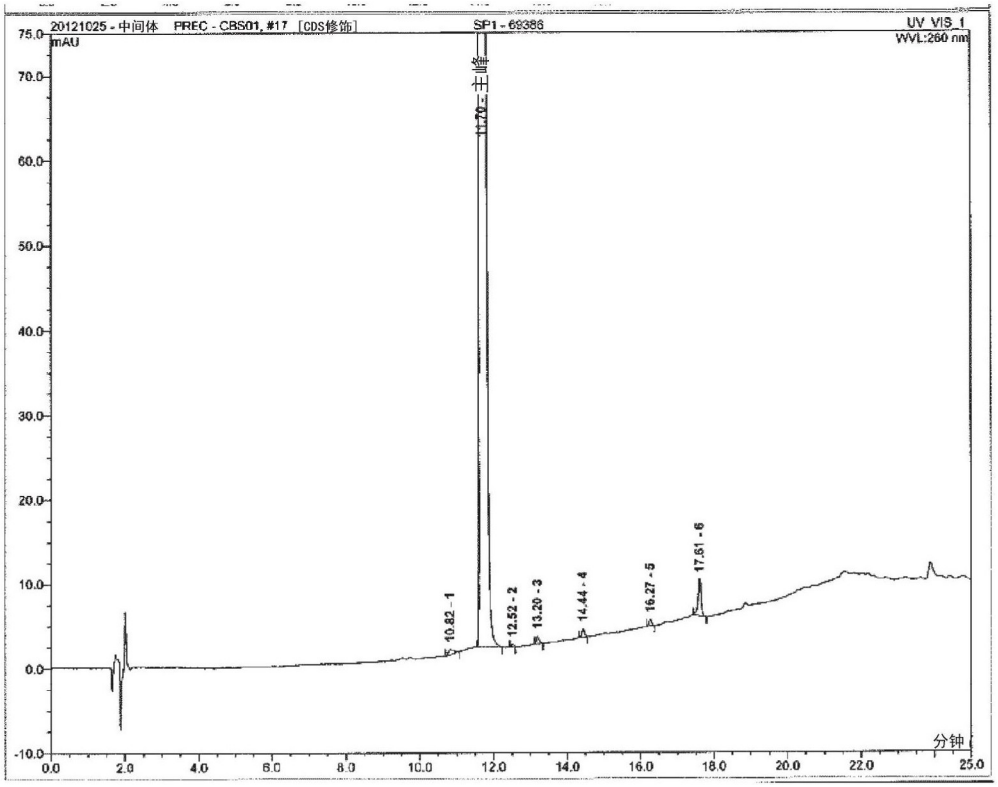

20121025 - 中间体 PREC - CBS01, #17 [CDS修饰]
SP1 - 69386
UV_VIS_1
WVL:260 nm
mAU
11.70 - 主峰
10.82 - 1
12.52 - 2
13.20 - 3
14.44 - 4
16.27 - 5
17.61 - 6
分钟

图2A

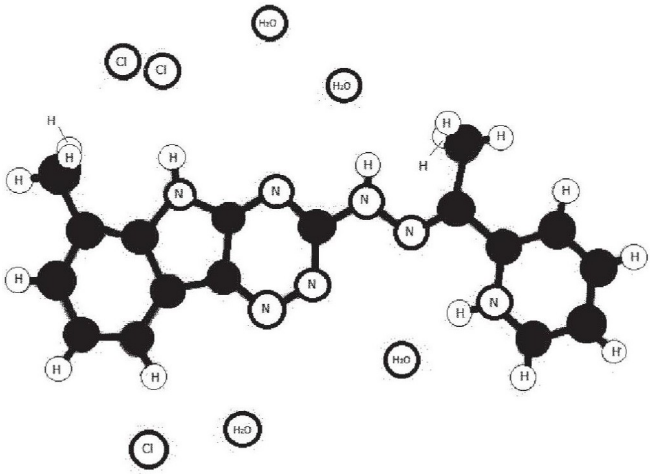


图2B

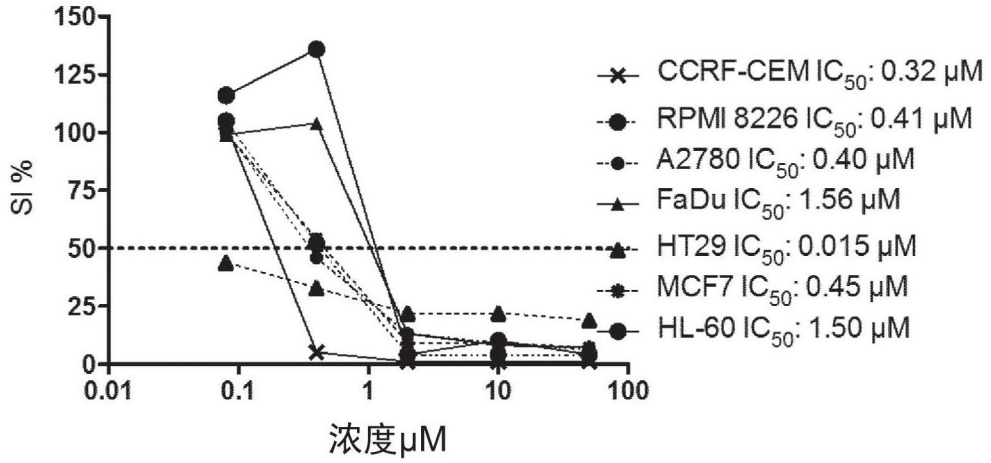
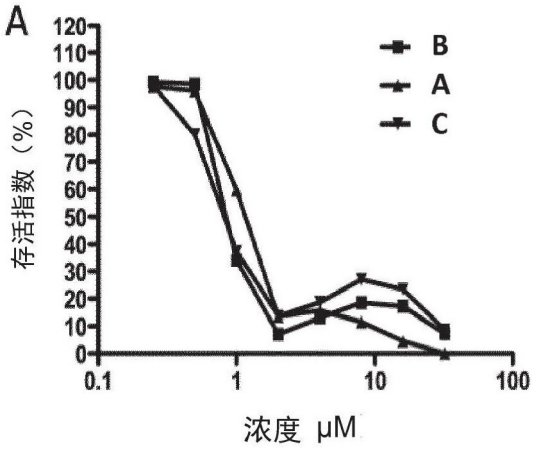
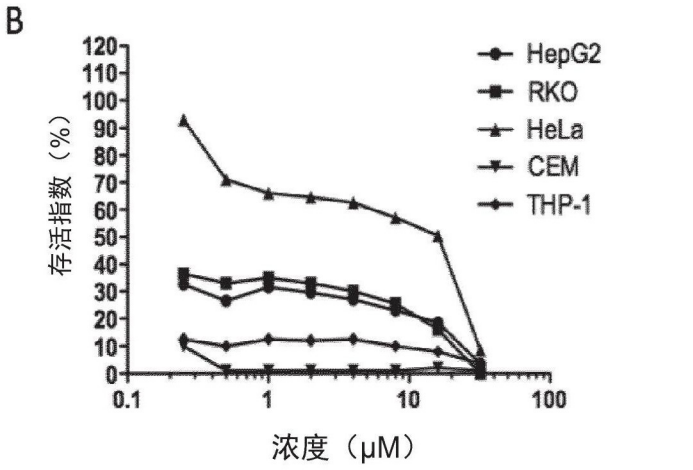
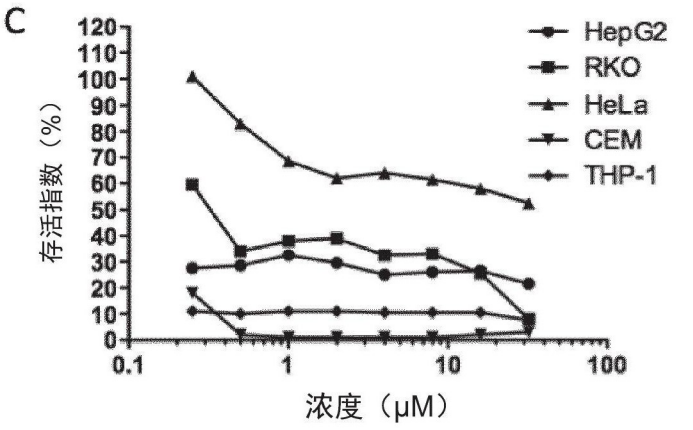

150
125
100
75
50
25
0
SI %
0.01
0.1
1
10
100
浓度μM
CCRF-CEM IC50: 0.32 μM
RPMI 8226 IC50: 0.41 μM
A2780 IC50: 0.40 μM
FaDu IC50: 1.56 μM
HT29 IC50: 0.015 μM
MCF7 IC50: 0.45 μM
HL-60 IC50: 1.50 μM

图3


A
120
110
100
90
80
70
60
50
40
30
20
10
0
存活指数（%）
0.1
1
10
100
浓度 μM
B
A
C


B
存活指数（%）
浓度（μM）
HepG2
RKO
HeLa
CEM
THP-1


C
存活指数（%）
浓度（μM）
HepG2
RKO
HeLa
CEM
THP-1

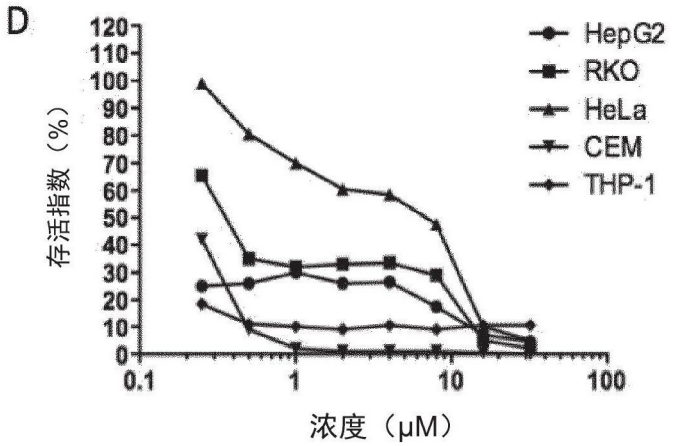

D
存活指数（%）
浓度（μM）
HepG2
RKO
HeLa
CEM
THP-1

图4