

[19] Patents Registry [11] 1255941 B
The Hong Kong Special Administrative Region
香港特別行政區
專利註冊處

[12] **STANDARD PATENT (R) SPECIFICATION**
轉錄標準專利說明書

[21] Application no. 申請編號 [51] Int. Cl.
18115010.4 C07D 515/22 (2006.01) A61P 35/00 (2006.01)
[22] Date of filing 提交日期 A61K 31/4995 (2006.01)
23.11.2018

[54] ANTITUMORAL COMPOUNDS
抗腫瘤化合物

[30] Priority 優先權 27.04.2017 EP 17382228 26.07.2017 EP 17382497	[73] Proprietor 專利所有人 Pharma Mar, S.A. Avenida de los Reyes, 1 Polígono Industrial La Mina, 28770 Colmenar Viejo, Madrid SPAIN
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[45] Date of publication of grant of patent 批予專利的發表日期 29.04.2020 EP Application no. & date 歐洲專利申請編號及日期 EP 18382288.1 27.04.2018 EP Publication no. & date 歐洲專利申請發表編號及日期 EP 3395821 31.10.2018 Date of grant in designated patent office 指定專利當局批予專利日期 15.05.2019	[74] Agent and / or address for service 代理人及/或送達地址 MARKS & CLERK Level 9, Cyberport 1 100 Cyberport Road, Pok Fu Lam HONG KONG



(19)

Europäisches
Patentamt
European
Patent Office
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des brevets



(11)

EP 3 395 821 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
15.05.2019 Bulletin 2019/20

(51) Int Cl.:
C07D 515/22 (2006.01) **A61P 35/00 (2006.01)**
A61K 31/4995 (2006.01)

(21) Application number: **18382288.1**

(22) Date of filing: **27.04.2018**

(54) **ANTITUMORAL COMPOUNDS**

ANTITUMORVERBINDUNGEN

COMPOSÉS ANTITUMORAUX

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME
Designated Validation States:
KH MA MD TN

(30) Priority: **27.04.2017 EP 17382228**
26.07.2017 EP 17382497

(43) Date of publication of application:
31.10.2018 Bulletin 2018/44

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WO-A1-03/014127 **WO-A1-2011/147828**

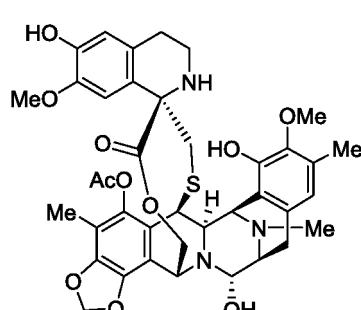
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Description**FIELD OF THE INVENTION**

5 [0001] The present invention relates to synthetic analogues of the ecteinascidins, particularly of ecteinascidin 736 (ET-736), pharmaceutical compositions containing them, methods for their manufacture and their use as antitumoral agents.

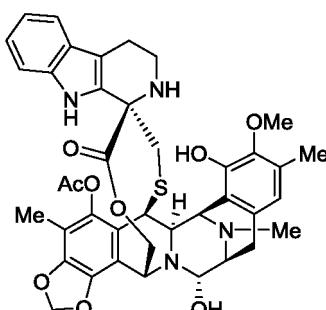
BACKGROUND OF THE INVENTION

10 [0002] The ecteinascidins are exceedingly potent antitumor agents isolated from the marine tunicate *Ecteinascidia turbinata*. One of these compounds, ET-743 of formula:



25 is being employed as an anticancer medicament, under the international nonproprietary name (INN) trabectedin, for the treatment of patients with advanced and metastatic soft tissue sarcoma (STS) after failure of anthracyclines and ifosfamide, or who are unsuited to receive such agents, and for the treatment of relapsed platinum-sensitive ovarian cancer in combination with pegylated liposomal doxorubicin.

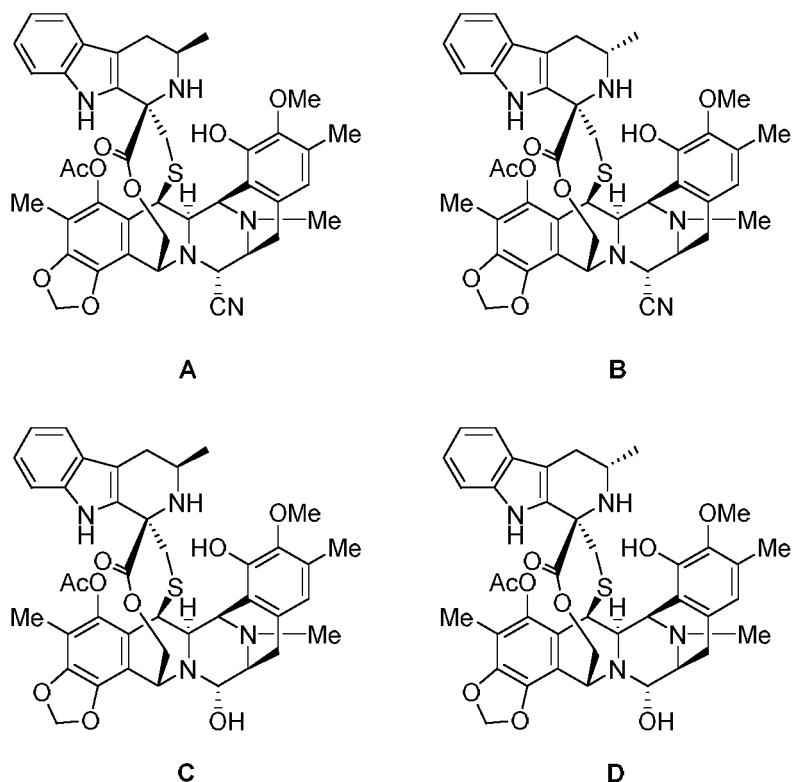
30 [0003] Ecteinascidin 736 (ET-736) was first discovered by Rinehart and features a tetrahydro-β-carboline unit in place of the tetrahydroisoquinoline unit more usually found in the ecteinascidin compounds isolated from natural sources; See for example Sakai et al., Proc. Natl. Acad. Sci. USA 1992, vol. 89, 11456-11460.



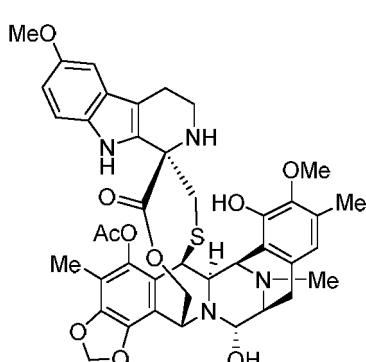
ET-736

45 [0004] U.S. Patent No. 5,149,804 describes Ecteinascidin 736 (ET-736), isolated from the Caribbean tunicate *Ecteinascidia turbinata*, and its structure. ET-736 protects mice *in vivo* at very low concentrations against P388 lymphoma, B16 melanoma, and Lewis lung carcinoma.

50 [0005] WO03014127 describes several synthetic analogues of ET-736 and their cytotoxic activity against tumoral cells. In particular, WO03014127 describes compounds **A** to **D** together with their cytotoxic activity against a panel of cancer cell lines.



[0006] Another compound described in this patent application, **PM01183**, is currently in clinical trials for the treatment of cancer. **PM01183** has the following chemical structure:



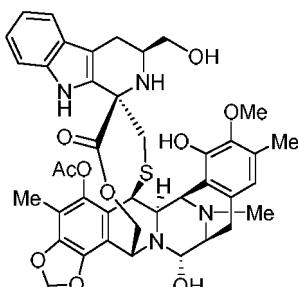
[0007] **PM01183** has demonstrated a highly potent *in vitro* activity against solid and non-solid tumour cell lines as well as a significant *in vivo* activity in several xenografted human tumor cell lines in mice, such as those for breast, kidney and ovarian cancer. **PM01183** exerts its anticancer effects through the covalent modification of guanines in the DNA minor groove that eventually give rise to DNA double-strand break, S-phase arrest and apoptosis in cancer cells.

[0008] WO2011147828 describes preparation procedures of ecteinascidin analogues which act as anticancer agents.

[0009] Despite the positive results obtained in clinical applications in chemotherapy, the search in the field of ecteinascidin compounds is still open to the identification of new compounds with optimal features of activity, selectivity toward the tumour, with a reduced systemic toxicity and/or improved pharmacokinetic properties.

SUMMARY OF THE INVENTION

[0010] In a first aspect of the present invention there is provided a compound of formula I or a pharmaceutically acceptable salt or ester thereof:



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[0011] In a further aspect of the present invention, there is provided a pharmaceutical composition comprising a compound according to the present invention and a pharmaceutically acceptable carrier.

[0012] In a yet further aspect of the present invention, there is provided a dosage form comprising a pharmaceutical composition according to the present invention.

[0013] In a yet further aspect of the present invention, there is provided a compound, a pharmaceutical composition or dosage form according to the present invention for use as a medicament.

[0014] In a yet further aspect of the present invention, there is provided a compound, a pharmaceutical composition or dosage form according to the present invention for use in the treatment of cancer.

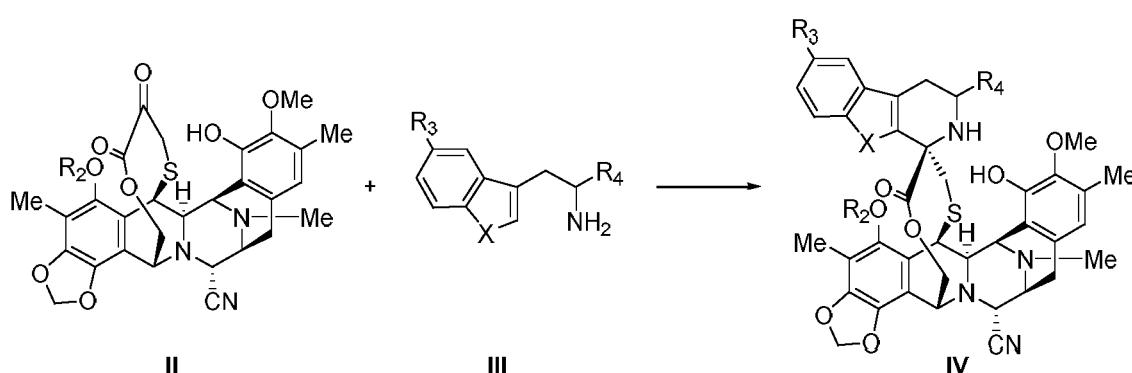
[0015] In a yet further aspect of the present invention, there is provided the use of a compound, pharmaceutical composition or dosage form according to the present invention for the manufacture of a medicament for the treatment of cancer.

[0016] Further disclosed is a method for the prevention or treatment of cancer, comprising administering an effective amount of a compound according to the present invention or administering an effective amount of a pharmaceutical composition according to the present invention, or administering an effective amount of a dosage form according to the present invention to a patient in need thereof, notably a human.

[0017] Further disclosed is the use of a compound according to the present invention for the treatment of cancer, or in the preparation of a medicament preferably for the treatment of cancer.

[0018] In a yet further aspect of the present invention, there is provided a kit comprising a therapeutically effective amount of a compound according to the present invention and a pharmaceutically acceptable carrier. The kit is for use in the treatment of cancer.

[0019] In a yet further aspect of the present invention, there is provided a process for obtaining the compound of formula I or a pharmaceutically acceptable salt or ester thereof; comprising the step of reacting a compound of formula II with a compound of formula III to give a compound of formula IV.



50 wherein:

X is -NH- ;
R₂ is acetyl;
R₃ is hydrogen;
R₄ is $\text{-CH}_2\text{OH}$.

[0020] The process may include the further step of replacing the cyano group in the compound of formula **IV** with a hydroxy group to give a compound of formula **I**.

BRIEF DESCRIPTION OF THE FIGURES

[0021]

5 **Figure 1.** Tumor total diameter evaluation of HT1080 tumors in mice treated with placebo, compound **C**, and **4-S**.
Figure 2. Tumor volume evaluation of MDA-MB-231 tumors in mice treated with placebo, compound **C**, and **4-S**.
Figure 3. Tumor volume evaluation of H460 tumors in mice treated with placebo, compound **C**, and **4-S**.
Figure 4. Tumor volume evaluation of H526 tumors in mice treated with placebo, compound **C**, and **4-S**.
Figure 5. Tumor volume evaluation of H82 tumors in mice treated with placebo, compound **C**, and **4-S**.
10 **Figure 6.** Tumor volume evaluation of A2780 tumors in mice treated with placebo, compound **C**, and **4-S**.
Figure 7. Tumor volume evaluation of HGC-27 tumors in mice treated with placebo, compound **C**, and **4-S**.
Figure 8. Tumor volume evaluation of PC-3 tumors in mice treated with placebo and **4-S**.
Figure 9. Tumor volume evaluation of DU-145 tumors in mice treated with placebo and **4-S**.
15 **Figure 10.** Tumor volume evaluation of 22Rv1 tumors in mice treated with placebo and **4-S**.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0022] The following apply to all aspects of the present invention:

The terms "pharmaceutically acceptable salt" and "ester" refers to any pharmaceutically acceptable salt or ester which, upon administration to the patient is capable of providing (directly or indirectly) a compound as described herein. However, it will be appreciated that non-pharmaceutically acceptable salts also fall within the scope of the invention since those may be useful in the preparation of pharmaceutically acceptable salts. The preparation of salts can be carried out by methods known in the art.

[0023] For instance, pharmaceutically acceptable salts of the compounds provided herein are synthesized from the parent compounds, which contain a basic or acidic moiety, by conventional chemical methods. Generally, such salts are, for example, prepared by reacting the free acid or base of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent or in a mixture of both. Generally, nonaqueous media like ether, ethyl acetate, ethanol, 2-propanol or acetonitrile are preferred. Examples of the acid addition salts include mineral acid addition salts such as, for example, hydrochloride, hydrobromide, hydroiodide, sulfate, nitrate, phosphate, and organic acid addition salts such as, for example, acetate, trifluoroacetate, maleate, fumarate, citrate, oxalate, succinate, tartrate, malate, mandelate, methanesulfonate and *p*-toluenesulfonate. Examples of the alkali addition salts include inorganic salts such as, for example, sodium, potassium, calcium and ammonium salts, and organic alkali salts such as, for example, ethylenediamine, ethanolamine, *N,N*-dialkylenethanolamine, triethanolamine and basic aminoacids salts.

[0024] The compounds of the invention may be in crystalline or amorphous form either as free compounds or as solvates (e.g. hydrates) and it is intended that all forms are within the scope of the present invention. Methods of solvation are generally known within the art.

[0025] Stereoisomerism about the asymmetric carbons with unspecified stereochemistry is possible, therefore in such cases the asymmetric carbons can have (*R*) or (*S*) configuration. All diastereomers generated by a specific configuration of such asymmetric carbons in conjunction with the other asymmetric carbons present in the molecule, and mixtures thereof, are considered within the scope of the present invention. Stereoisomerism about the double bond (geometric isomerism) is also possible, therefore in some cases the molecule could exist as (*E*)-isomer or (*Z*)-isomer. If the molecule contains several double bonds, each double bond will have its own stereoisomerism, that could be the same or different than the stereoisomerism of the other double bonds of the molecule. Furthermore, compounds referred to herein may exist as atropoisomers. The single stereoisomers including diastereoisomers, geometric isomers and atropoisomers of the compounds referred to herein, and mixtures thereof fall within the scope of the present invention.

[0026] In addition, compounds referred to herein may exist in isotopically-labelled forms. All pharmaceutically acceptable salts, esters and isotopically labelled forms of the compounds referred to herein, and mixtures thereof, are considered within the scope of the present invention.

[0027] To provide a more concise description, some of the quantitative expressions given herein are not qualified with the term "about". It is understood that, whether the term "about" is used explicitly or not, every quantity given herein is meant to refer to the actual given value, and it is also meant to refer to the approximation to such given value that would reasonably be inferred based on the ordinary skill in the art, including equivalents and approximations due to the experimental and/or measurement conditions for such given value.

[0028] An important feature of the above-described compound is its bioactivity and in particular its cytotoxic activity. In this regard, we have surprisingly found that the compound of the present invention shows an enhanced antitumor activity, as it is shown in Examples 3, 5 and 6..

[0029] In a further embodiment of the present invention, there is provided a pharmaceutical composition comprising

a compound according to the present invention and a pharmaceutically acceptable carrier. Examples of the administration form include without limitation oral, topical, parenteral, sublingual, rectal, vaginal, ocular and intranasal. Parenteral administration includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. Preferably the compositions are administered parenterally. Pharmaceutical compositions of the invention can be formulated so as to allow a compound according to the present invention to be bioavailable upon administration of the composition to an animal, preferably human. Compositions can take the form of one or more dosage units, where for example, a tablet can be a single dosage unit, and a container of a compound according to the present invention may contain the compound in liquid or in aerosol form and may hold a single or a plurality of dosage units.

[0030] The pharmaceutically acceptable carrier or vehicle can be particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) can be liquid, with the compositions being, for example, an oral syrup or injectable liquid. In addition, the carrier(s) can be gaseous, or liquid so as to provide an aerosol composition useful in, for example inhalatory administration. Powders may also be used for inhalation dosage forms. The term "carrier" refers to a diluent, adjuvant or excipient, with which the compound according to the present invention is administered. Such pharmaceutical carriers can be liquids, such as water and oils including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The carriers can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, disaccharides, and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents can be used. In one embodiment, when administered to an animal, the compounds and compositions according to the present invention, and pharmaceutically acceptable carriers are sterile. Water is a preferred carrier when the compounds according to the present invention are administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

[0031] When intended for oral administration, the composition is preferably in solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

[0032] As a solid composition for oral administration, the composition can be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition typically contains one or more inert diluents. In addition, one or more for the following can be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, corn starch and the like; lubricants such as magnesium stearate; glidants such as colloidal silicon dioxide; sweetening agent such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; and a coloring agent.

[0033] When the composition is in the form of a capsule (e.g. a gelatin capsule), it can contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol, cyclodextrins or a fatty oil.

[0034] The composition can be in the form of a liquid, e.g. an elixir, syrup, solution, emulsion or suspension. The liquid can be useful for oral administration or for delivery by injection. When intended for oral administration, a composition can comprise one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition for administration by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent can also be included.

[0035] The preferred route of administration is parenteral administration including, but not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, intracerebral, intraventricular, intrathecal, intravaginal or transdermal. The preferred mode of administration is left to the discretion of the practitioner, and will depend in part upon the site of the medical condition (such as the site of cancer). In a more preferred embodiment, the compound according to the present invention is administered intravenously. Infusion times of up to 24 hours are preferred to be used, more preferably 1 to 12 hours, with 1 to 6 hours being most preferred. Short infusion times which allow treatment to be carried out without an overnight stay in a hospital are especially desirable. However, infusion may be 12 to 24 hours or even longer if required. Infusion may be carried out at suitable intervals of, for example, 1 to 4 weeks.

[0036] The liquid compositions of the invention, whether they are solutions, suspensions or other like form, can also include one or more of the following: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides, polyethylene glycols, glycerin, or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; and agents for the adjustment of tonicity such as sodium chloride or dextrose. A parenteral composition can be enclosed in an ampoule, a disposable syringe or a multiple-dose vial made of glass, plastic or other material. Physiological saline is a preferred adjuvant.

[0037] The amount of the compound according to the present invention that is effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays can optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the compositions will also depend on the route of administration, and the seriousness

of the disease or disorder, and should be decided according to the judgement of the practitioner and each patient's circumstances.

[0038] The compositions comprise an effective amount of a compound of the present invention such that a suitable dosage will be obtained. The correct dosage of the compounds will vary according to the particular formulation, the mode of application, and its particular site, host and the disease being treated, e.g. cancer and, if so, what type of tumor. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease should be taken into account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

[0039] Typically, the amount is at least about 0.01% of a compound of the present invention, and may comprise at least 80%, by weight of the composition. When intended for oral administration, this amount can be varied to range from about 0.1% to about 80% by weight of the composition. Preferred oral compositions can comprise from about 4% to about 50% of the compound of the present invention by weight of the composition.

[0040] Preferred compositions of the present invention are prepared so that a parenteral dosage unit contains from about 0.01% to about 10 % by weight of the compound of the present invention. More preferred parenteral dosage unit contains about 0.5 % to about 5 % by weight of the compound of the present invention.

[0041] For intravenous administration, the composition is suitable for doses from about 0.1 mg/kg to about 250 mg/kg of the animal's body weight, preferably from about 0.1 mg/kg and about 20 mg/kg of the animal's body weight, and more preferably from about 1 mg/kg to about 10 mg/kg of the animal's body weight.

[0042] The compound of the present invention, can be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings.

[0043] In specific embodiments, it can be desirable to administer the compound of the present invention, or compositions locally to the area in need of treatment. In one embodiment, administration can be by direct injection at the site (or former site) of a cancer, tumor or neoplastic or pre-neoplastic tissue.

[0044] Pulmonary administration can also be employed, e.g. by use of an inhaler or nebulizer, and formulation with an aerosolizing agent, or via perfusion in a fluorocarbon or synthetic pulmonary surfactant. In certain embodiments, the compound of the present invention can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

[0045] The present compositions can take the form of solutions, suspensions, emulsions, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. Other examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

[0046] The pharmaceutical compositions can be prepared using methodology well known in the pharmaceutical art. For example, a composition intended to be administered by injection can be prepared by combining a compound of the present invention with water, or other physiologically suitable diluent, such as phosphate buffered saline, so as to form a solution. A surfactant can be added to facilitate the formation of a homogeneous solution or suspension. Preferred compositions according to the present invention include:

- Pharmaceutical compositions comprising a compound of the present invention and a disaccharide. Particularly preferred disaccharides are selected from lactose, trehalose, sucrose, maltose, isomaltose, cellobiose, isosaccharose, isotrehalose, turanose, melibiose, gentiobiose, and mixtures thereof.
- Lyophilised pharmaceutical compositions comprising a compound of the present invention and a disaccharide. Particularly preferred disaccharides are selected from lactose, trehalose, sucrose, maltose, isomaltose, cellobiose, isosaccharose, isotrehalose, turanose, melibiose, gentiobiose, and mixtures thereof.

[0047] The ratio of the active substance to the disaccharide in embodiments of the present invention is determined according to the solubility of the disaccharide and, when the formulation is freeze dried, also according to the freeze-dryability of the disaccharide. It is envisaged that this active substance:disaccharide ratio (w/w) can be about 1:10 in some embodiments, about 1:20 in other embodiments, about 1:50 in still other embodiments. It is envisaged that other embodiments have such ratios in the range from about 1:5 to about 1:500, and still further embodiments have such ratios in the range from about 1:10 to about 1:500.

[0048] The composition comprising a compound of the present invention may be lyophilized. The composition comprising a compound of the present invention is usually presented in a vial which contains a specified amount of such compound.

[0049] We have found that the compound of the present invention and compositions of the present invention are particularly effective in the treatment of cancer.

[0050] The present invention provides a compound or composition for use as medicament. The present invention provides a compound or composition for use in the treatment of cancer, and more preferably a cancer selected from

lung cancer, including non-small cell lung cancer and small cell lung cancer, colon cancer, breast cancer, pancreas cancer, sarcoma, ovarian cancer and gastric cancer.

[0051] Thus, the compound and compositions according to the present invention are useful for inhibiting the multiplication, or proliferation, of a tumor cell or cancer cell, or for treating cancer in an animal.

[0052] The compounds and compositions according to the present invention show excellent activity in the treatment of cancers such as lung cancer including non-small cell lung cancer and small cell lung cancer, colon cancer, breast cancer, pancreas cancer, sarcoma, ovarian cancer and gastric cancer. Most preferred cancers are selected from lung cancer including non-small cell lung cancer and small cell lung cancer, breast cancer, pancreas cancer and colorectal cancer.

[0053] In the present application, by "cancer" it is meant to include tumors, neoplasias and any other malignant disease having as cause malignant tissue or cells.

[0054] The term "treating", as used herein, unless otherwise indicated, means reversing, attenuating, alleviating or inhibiting the progress of the disease or condition to which such term applies, or one or more symptoms of such disorder or condition. The term "treatment", as used herein, unless otherwise indicated, refers to the act of treating as "treating" is defined immediately above.

[0055] The compound and compositions according to the present invention can be administered to an animal that has also undergone surgery as treatment for the cancer. In one embodiment of the present invention, the additional method of treatment is radiation therapy.

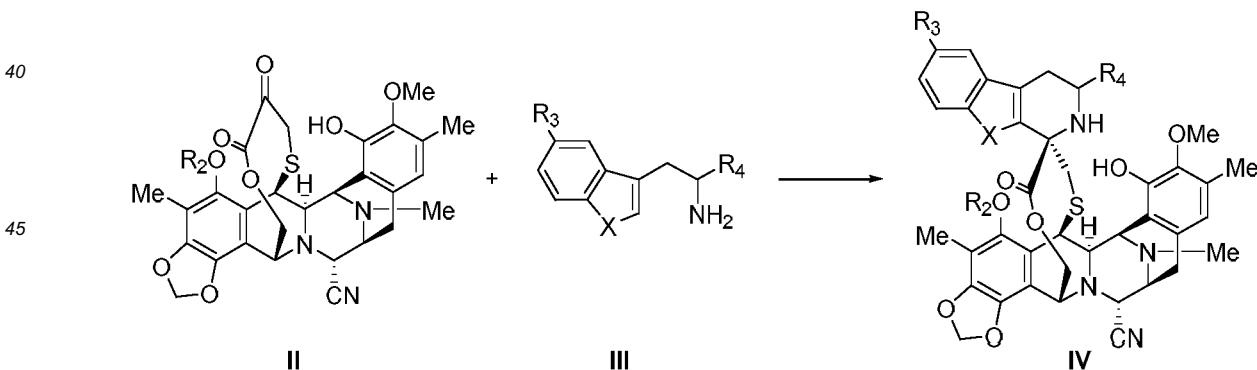
[0056] In a specific embodiment of the present invention, the compound or composition according to the present invention is administered concurrently with radiation therapy. In another specific embodiment, the radiation therapy is administered prior or subsequent to administration of the compound or composition of the present invention, preferably at least an hour, three hours, five hours, 12 hours, a day, a week, a month, more preferably several months (e.g. up to three months) prior or subsequent to administration of a compound or composition of the present invention.

[0057] Any radiation therapy protocol can be used depending upon the type of cancer to be treated. For example, but not by way of limitation, x-ray radiation can be administered; in particular, high-energy megavoltage (radiation of greater than 1 MeV energy) can be used for deep tumors, and electron beam and orthovoltage x-ray radiation can be used for skin cancers. Gamma-ray emitting radioisotopes, such as radioactive isotopes of radium, cobalt and other elements, can also be administered.

[0058] In a further embodiment of the present invention, there is provided a kit comprising a therapeutically effective amount of a compound according to the present invention and a pharmaceutically acceptable carrier.

[0059] In one embodiment, the kit according to this embodiment is for use in the treatment of cancer, and more preferably a cancer selected from lung cancer, including non-small cell lung cancer and small cell lung cancer, colon cancer, breast cancer, pancreas cancer, sarcoma, ovarian cancer and gastric cancer.

[0060] In a further embodiment of the present invention, there is provided a process for obtaining a compound of formula I or a pharmaceutically acceptable salt or ester thereof, comprising the step of reacting a compound of formula II with a compound of formula III to give a compound of formula IV:



X is -NH-;
R₂ is acetyl;
R₃ is hydrogen;
R₄ is -CH₂OH.

[0061] In a more preferred embodiment, the process further comprises the step of replacing the cyano group in the

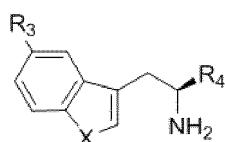
compound of formula **IV** with a hydroxy group to give a compound of formula **I**.

[0062] Preferred processes according to the present invention include:

- A process wherein the compound of formula **III** is a compound of formula **IIIa**:

5

10



IIIa

wherein

15

X is -NH- ;
R₃ is hydrogen; and
R⁴ is -CH₂OH.

EXAMPLES

[0063] Compound **1** was prepared as described in Example 20 of WO 01/87895.

[0064] Reference compound **C** was prepared as described in WO 03/014127 (Compound **44**).

25 Example 1.

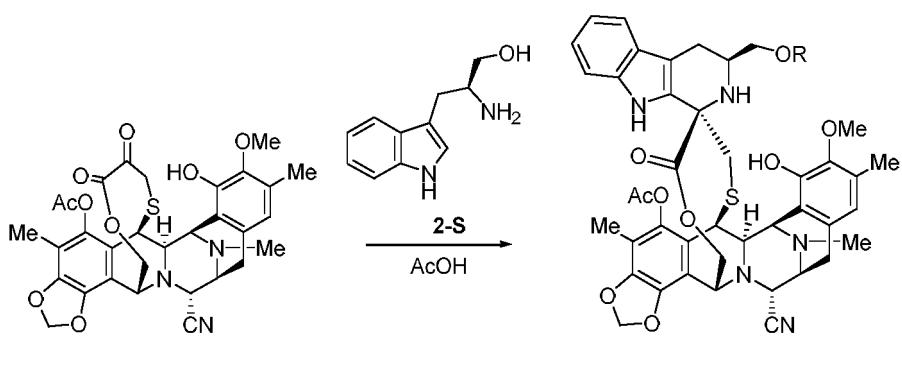
A)

[0065]

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[0066] To a solution of **1** (0.5 g, 0.80 mmol) in acetic acid (20 mL, 0.04 M) was added *L*-tryptophanol (**2-S**) (533 mg, 3.0 mmol, Sigma-Aldrich). The reaction mixture was stirred at 23 °C for 16 h and then acetic acid was evaporated. An aqueous saturated solution of NaHCO₃ was added and the mixture was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum. Flash chromatography (Hexane:EtOAc, 1:1) gave compounds **3-S** (616 mg, 97%) and **3a-S** (12 mg, 2%).

50

3-S

[0067] R_f = 0.50 (Hexane:EtOAc, 1:1).

¹H NMR (300 MHz, CDCl₃): δ 7.71 (s, 1H), 7.36 (dd, J = 7.9, 1.0 Hz, 1H), 7.27 (dd, J = 8.2, 0.9 Hz, 1H), 7.13 (ddd, J = 8.3, 7.0, 1.2 Hz, 1H), 7.03 (ddd, J = 8.0, 7.0, 1.0 Hz, 1H), 6.62 (s, 1H), 6.26 (d, J = 1.4 Hz, 1H), 6.04 (d, J = 1.3 Hz, 1H), 5.75 (s, 1H), 5.14 (dd, J = 11.7, 1.2 Hz, 1H), 4.60 (s, 1H), 4.41 (s, 1H), 4.36-4.24 (m, 2H), 4.21 (d, J = 2.7 Hz, 1H), 3.82 (s, 3H), 3.52 (s, 1H), 3.50-3.47 (m, 1H), 3.45 (dq, J = 8.4, 2.2 Hz, 1H), 3.35 (t, J = 10.1 Hz, 1H), 3.01-2.78 (m, 5H), 2.62 (dd, J = 15.3, 4.7 Hz, 1H), 2.41 (s, 1H), 2.38 (s, 3H), 2.37-2.31 (m, 1H), 2.28 (s, 3H), 2.17 (s, 3H), 2.06 (s, 3H).

ESI-MS *m/z*: 794.2 (M+H)⁺.

3a-S

[0068] R_f = 0.70 (Hexane:EtOAc, 1:1).

¹H NMR (500 MHz, CDCl₃): δ 7.83 (s, 1H), 7.38 (dt, *J* = 7.9, 0.9 Hz, 1H), 7.25 (dt, *J* = 8.3, 0.9 Hz, 1H), 7.11 (ddd, *J* = 8.2, 7.1, 1.2 Hz, 1H), 7.02 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 6.62 (s, 1H), 6.24 (d, *J* = 1.4 Hz, 1H), 6.03 (d, *J* = 1.3 Hz, 1H), 5.79 (s, 1H), 5.13 (d, *J* = 11.7 Hz, 1H), 4.60 (s, 1H), 4.39 (s, 1H), 4.36-4.22 (m, 3H), 4.17-4.09 (m, 1H), 3.91 (dd, *J* = 10.5, 8.6 Hz, 1H), 3.83 (s, 3H), 3.51-3.41 (m, 2H), 3.04-2.92 (m, 3H), 2.72 (dd, *J* = 15.1, 4.0 Hz, 1H), 2.54-2.41 (m, 2H), 2.38 (s, 3H), 2.35-2.30 (m, 1H), 2.29 (s, 3H), 2.21-2.16 (m, 1H), 2.18 (s, 3H), 2.12 (s, 3H); 2.05 (s, 3H).
¹³C NMR (101 MHz, CDCl₃): δ 171.2, 170.7, 168.6, 147.5, 145.8, 143.0, 141.1, 140.4, 135.6, 130.1, 129.5, 126.7, 122.2, 121.2, 120.9, 119.4, 118.4, 118.2, 118.2, 113.6, 113.5, 110.9, 110.0, 109.1, 102.1, 91.4, 67.2, 63.4, 61.3, 60.4, 59.7, 59.1, 54.8, 54.6, 47.7, 42.0, 41.6, 31.6, 24.0, 22.6, 21.0, 15.9, 14.2, 9.7.

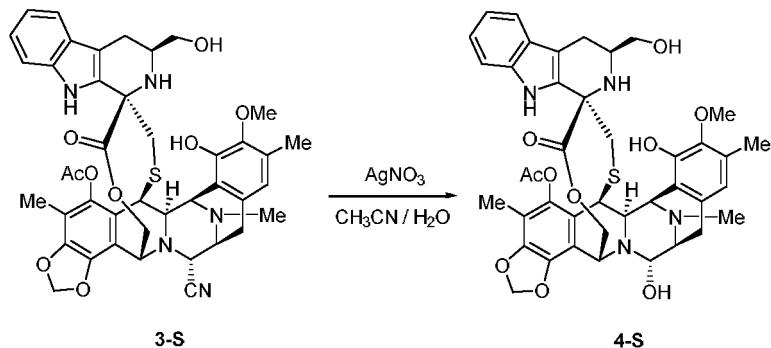
ESI-MS *m/z*: 836.2 (M+H)⁺.

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B)

[0069]

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[0070] To a solution of **3-S** (616 mg, 0.77 mmol) in CH₃CN:H₂O (1.39:1, 51 mL, 0.015 M) was added AgNO₃ (3.40 g, 23.3 mmol). After 3 h at 23 °C, the reaction mixture was quenched with a mixture 1:1 of saturated aqueous solutions of NaCl and NaHCO₃, stirred for 15 min, diluted with CH₂Cl₂, stirred for 5 min, and extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄, filtered, and concentrated under vacuum. The residue obtained was purified by flash chromatography (CH₂Cl₂:CH₃OH, from 99:1 to 85:15) to give **4-S** (471 mg, 78%).

[0071] R_f = 0.50 (CH₂Cl₂:CH₃OH, 9:1).

¹H NMR (500 MHz, CDCl₃): δ 7.71 (s, 1H), 7.36 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.26 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.12 (ddd, *J* = 8.2, 7.0, 1.2 Hz, 1H), 7.03 (ddd, *J* = 8.0, 7.1, 1.0 Hz, 1H), 6.64 (s, 1H), 6.23 (d, *J* = 1.3 Hz, 1H), 6.01 (d, *J* = 1.4 Hz, 1H), 5.75 (s, 1H), 5.25 (d, *J* = 11.4 Hz, 1H), 4.92 (s, 1H), 4.52 (br s, 3H), 4.22 (dd, *J* = 11.4, 2.2 Hz, 1H), 4.19 (s, 1H), 3.83 (s, 3H), 3.54 (br s, 2H), 3.35 (t, *J* = 10.2 Hz, 1H), 3.26 (s, 1H), 3.01-2.93 (m, 3H), 2.88 (br s, 3H), 2.63 (dd, *J* = 15.2, 4.8 Hz, 1H), 2.38 (s, 3H), 2.36-2.31 (m, 2H), 2.28 (s, 3H), 2.05 (s, 3H).

¹³C NMR (126 MHz, CDCl₃): δ 171.9, 168.6, 147.5, 145.4, 142.9, 141.2, 140.7, 135.5, 130.4, 126.8, 122.3, 122.0, 121.3, 119.4, 118.4, 115.2, 112.8, 111.0, 110.0, 109.6, 101.8, 81.9, 76.8, 65.2, 62.8, 62.5, 60.4, 58.1, 57.9, 55.9, 55.1, 53.4, 51.6, 41.8, 41.3, 39.6, 24.1, 23.8, 20.5, 15.8, 9.7.

ESI-MS *m/z*: 767.3 (M-H₂O+H)⁺.

(+)-HR-ESI-TOF-MS *m/z* 767.2788 [M-H₂O+H]⁺ (Calcd. for C₄₁H₄₃N₄O₉S: 767.2745).

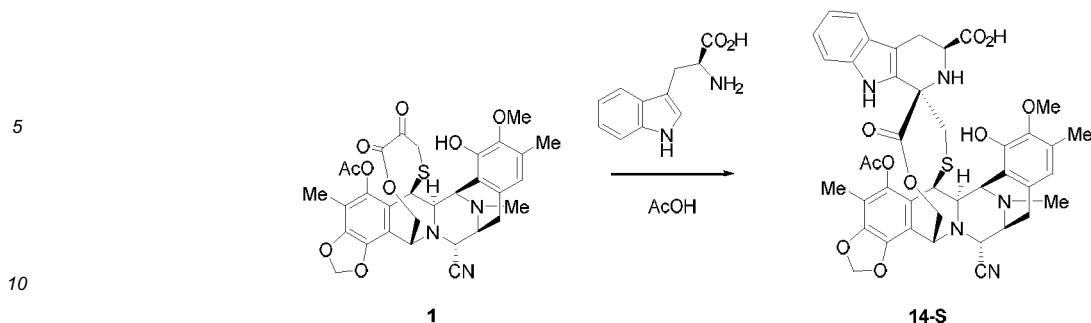
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Example 2. Synthesis of Reference Compounds 14-S and 15-S

A)

[0072]

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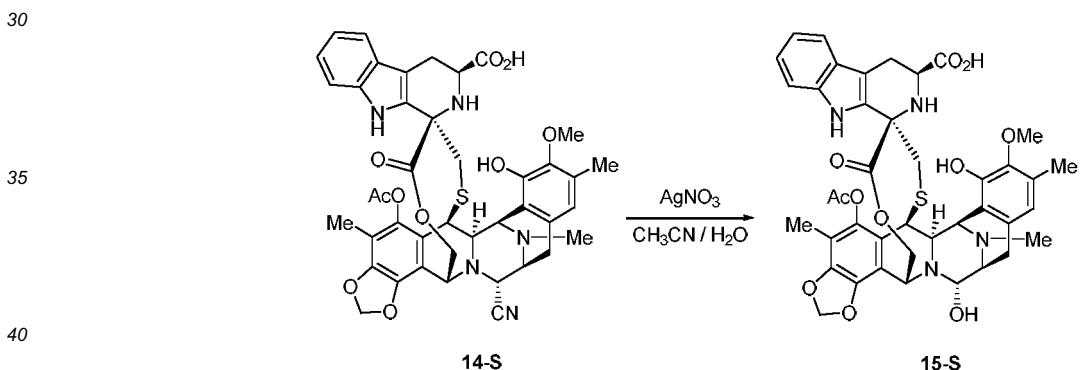
15 [0073] To a solution of **1** (50 mg, 0.08 mmol) in acetic acid (1 mL, 0.08 M) was added L-tryptophan (50 mg, 0.24 mmol). The reaction mixture was stirred at 50 °C for 17 h and then acetic acid was evaporated. An aqueous saturated solution of NaHCO₃ was added and the mixture was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum. Flash chromatography (CH₂Cl₂:CH₃OH, from 99:1 to 80:20) gave compound **14-S** (58 mg, 90%).

20 [0074] R_f = 0.20 (CH₂Cl₂:CH₃OH, 10:1).
¹H NMR (400 MHz, CDCl₃): δ 7.77 (s, 1H), 7.39 (d, J = 7.9 Hz, 1H), 7.25 (d, J = 7.9 Hz, 1H), 7.13 (ddd, J = 8.2, 7.0, 1.2 Hz, 1H), 7.04 (td, J = 7.5, 7.1, 1.0 Hz, 1H), 6.56 (s, 1H), 6.24 (d, J = 1.3 Hz, 1H), 6.03 (d, J = 1.3 Hz, 1H), 5.15 (d, J = 11.7 Hz, 1H), 4.62 (s, 1H), 4.43 (s, 1H), 4.35 (dd, J = 11.7, 2.1 Hz, 1H), 4.28 (dd, J = 5.2, 1.6 Hz, 1H), 4.20 (s, 1H), 3.78 (s, 3H), 3.52-3.41 (m, 4H), 3.07-2.88 (m, 2H), 2.91-2.80 (m, 2H), 2.42-2.21 (m, 2H), 2.35 (s, 3H), 2.27 (s, 3H), 2.14 (s, 3H), 2.04 (s, 3H).
ESI-MS m/z: 808.6 (M+H)⁺.

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B)

[0075]



45 [0076] To a solution of **14-S** (52 mg, 0.066 mmol) in CH₃CN:H₂O (2:1, 4.5 mL, 0.015 M) was added AgNO₃ (164 mg, 1.45 mmol). After 20 h at 23 °C, a mixture 1:1 of saturated aqueous solutions of NaCl and NaHCO₃ was added, stirred for 15 min, diluted with CH₂Cl₂, stirred for 30 min, and extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum. The residue obtained was purified by flash chromatography (CH₂Cl₂:CH₃OH, from 99:1 to 70:30) to afford **15-S** (18 mg, 35%).

50 [0077] R_f = 0.15 (CH₂C₁₂:CH₃OH, 9:1).
¹H NMR (400 MHz, CD₃OD): δ 7.76 (s, 1H), 7.40 (d, J = 7.8 Hz, 1H), 7.25 (d, J = 7.8 Hz, 1H), 7.14 (t, J = 7.4 Hz, 1H), 7.04 (t, J = 7.4 Hz, 1H), 6.58 (s, 1H), 6.23 (d, J = 1.3 Hz, 1H), 6.01 (d, J = 1.3 Hz, 1H), 5.28 (d, J = 12.7 Hz, 1H), 4.95 (s, 1H), 4.53 (s, 1H), 4.28 (dd, J = 11.4, 2.0 Hz, 1H), 4.21 (s, 1H), 3.80 (s, 3H), 3.58 (s, 1H), 3.52-3.47 (m, 2H), 3.28 (s, 1H), 3.03 (dd, J = 15.8, 5.2 Hz, 1H), 2.91-2.82 (m, 3H), 2.44 (d, J = 15.4 Hz, 1H), 2.36 (s, 3H), 2.35-2.31 (m, 1H), 2.28 (s, 3H), 2.15 (s, 3H), 2.03 (s, 3H).

55 ¹³C NMR (101 MHz, CDCl₃): δ 173.7, 171.2, 168.7, 147.5, 145.7, 142.8, 141.2, 140.8, 135.6, 129.8, 126.3, 122.8, 121.5, 121.2, 119.9, 118.6, 117.7, 115.0, 111.1, 101.9, 81.5, 66.8, 62.9, 60.4, 57.9, 55.8, 55.1, 52.3, 42.3, 41.3, 38.3, 31.9, 29.4, 28.9, 24.5, 24.0, 23.8, 22.7, 20.5, 16.0, 9.7.

ESI-MS m/z: 781.6 (M-H₂O+H)⁺.(+)-HR-ESI-TOF-MS m/z: 781.2610 [M-H₂O+H]⁺ (Calcd. for C₄₁H₄₁N₄O₁₀S: 781.2538).

Example 3. *In vitro* bioassays for the detection of antitumor activity

[0078] The aim of this assay is to evaluate the *in vitro* cytostatic (ability to delay or arrest tumor cell growth) or cytotoxic (ability to kill tumor cells) activity of the samples being tested.

CELL LINES					
	Name	Nº ATCC	Species	Tissue	Characteristics
	A549	CCL-185	human	lung	lung carcinoma (NSCLC)
10	HT29	HTB-38	human	colon	colorectal adenocarcinoma
	MDA-MB-231	HTB-26	human	breast	breast adenocarcinoma
15	PSN1	CRM-CRL-3211	human	pancreas	pancreas adenocarcinoma
	PC-3	CRL-1435	human	prostate	prostate adenocarcinoma
	22Rv1	CRL-2505	human	prostate	prostate carcinoma

EVALUATION OF CYTOTOXIC ACTIVITY USING THE SBR AND THE MTT COLORIMETRIC ASSAYS

[0079] A colorimetric assay, using sulforhodamine B (SRB) reaction has been adapted to provide a quantitative measurement of cell growth and viability (following the technique described by Skehan et al. J. Natl. Cancer Inst. 1990, 82, 1107-1112). Another colorimetric assay based on 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a purple formazan has been also used to assess the antiproliferative activity (following the technique described by Mosmann et al. J. Immunol. Meth. 1983, 65, 55-63).

[0080] These forms of assays employ 96-well cell culture microplates following the standards of the American National Standards Institute and the Society for Laboratory Automation and Screening (ANSI SLAS 1-2004 (R2012) 10/12/2011. All the cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and derive from different types of human cancer.

[0081] A549, HT29, MDA-MB-231 and PSN1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) while PC-3 and 22Rv1 cells were maintained in Roswell Park Memorial Institute Medium (RPMI). All cell lines were supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C, 5% CO₂ and 98% humidity. For the experiments, cells were harvested from subconfluent cultures using trypsinization and resuspended in fresh medium before counting and plating.

[0082] A549, HT29, MDA-MB-231 and PSN1 cells were seeded in 96 well microtiter plates, at 5000 cells per well in aliquots of 150 µL, and allowed to attach to the plate surface for 18 hours (overnight) in drug free medium. After that, one control (untreated) plate of each cell line was fixed (as described below) and used for time zero reference value. Culture plates were then treated with test compounds (50 µL aliquots of 4X stock solutions in complete culture medium plus 4% DMSO) using ten 2/5 serial dilutions (concentrations ranging from 10 to 0.003 µg/mL) and triplicate cultures (1% final concentration in DMSO). After 72 hours treatment, the antitumor effect was measured by using the SRB methodology: Briefly, cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde solution at room temperature, rinsed twice in PBS, and stained in 0.4% SRB solution for 30 min at room temperature. Cells were then rinsed several times with 1% acetic acid solution and air-dried at room temperature. SRB was then extracted in 10 mM trizma base solution and the absorbance measured in an automated spectrophotometric plate reader at 490 nm.

[0083] An appropriate number of PC-3 and 22Rv1 cells, to reach a final cell density in the assay ranging from 5,000 to 15,000 cells per well depending on the cell line, were seeded in 96-well plates and allowed to stand in culture medium for 24 h at 37°C under 5% CO₂ and 98% humidity. Then, compounds or DMSO in culture medium were added to reach a final volume of 200 µL and the intended compound concentration in a range covering ten serial 2/5 dilutions starting from 0.1 µg/mL in 1% (v/v) DMSO. At this point a set of "time zero control plates" treated with 1% (v/v) DMSO were processed with MTT as described below. The rest of the plates were incubated during 72 h under the aforementioned environmental conditions. Afterwards 50 µL of a 1 mg/mL MTT solution in culture medium were added to the wells and incubated for 6-8 hours at 37°C to allow formazan crystals generation. Culture medium was then removed and 100 µL of neat DMSO added to each well to dissolve the formazan product into a coloured solution whose absorbance at 540 nm was finally measured in a PolarStar Omega microplate multilabel reader (BMG Labtech, Ortenberg, Germany).

[0084] Effects on cell growth and survival were estimated by applying the NCI algorithm (Boyd MR and Paull KD. Drug Dev. Res. 1995, 34, 91-104). The values obtained in triplicate cultures were fitted by nonlinear regression to a four-parameters logistic curve by nonlinear regression analysis. Three reference parameters were calculated (according to the aforementioned NCI algorithm) by automatic interpolation of the curves obtained by such fitting: GI₅₀ = compound

concentration that produces 50% cell growth inhibition, as compared to control cultures; TGI = total cell growth inhibition (cytostatic effect), as compared to control cultures, and LC₅₀ = compound concentration that produces 50% net cell killing cytotoxic effect).

5 [0085] Table 1 illustrates data on the biological activity of the compound of the present invention together with biological activity of the reference compound. Table 2 provides data on the biological activity compared to an analogue with a carboxylic acid group. Compounds **C** and **15-S** are not part of the present invention.

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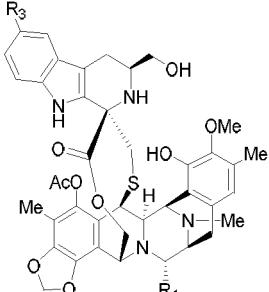
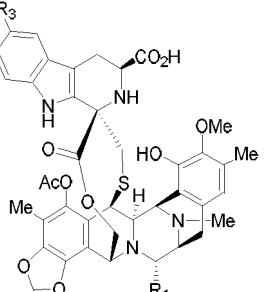
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Table 1. Biological activity (Molar)

Compound	Reference compound						
	C R ₁ = OH						
							
4-S R ₁ = OH, R ₄ = -CH ₂ OH	A549	HT29	MDA-MB-231	PSN1	PC-3	22Rv1	
GI₅₀	1.27E-09	1.27E-09	1.22E-09	1.78E-09	8.08E-10	3.58E-10	C 2.73E-08
TGI	4-S	1.40E-09	1.40E-09	2.55E-09	2.29E-09		C 6.63E-08
LC₅₀	>1.27E-07	>1.27E-07	6.50E-09	3.44E-09			>1.30E-07
							>1.30E-07
							6.50E-08

Table 2. Biological activity (Molar)

5	Compound					Reference compound				
	 <p>4-S R₁ = OH, R₃ = H</p>					 <p>15-S R₁ = OH, R₃ = H</p>				
10		A549	HT29	MDA-MB-231	PSN1		A549	HT29	MDA-MB-231	PSN1
15	GI₅₀ TGI LC₅₀	1.27E-09	1.27E-09	1.22E-09	1.78E-09	15-S	>1.25E-06	3.00E-07	1.63E-07	2.38E-07
20		1.40E-09	1.40E-09	2.55E-09	2.29E-09		>1.25E-06	5.13E-07	2.13E-07	4.63E-07
25		>1.27E-07	>1.27E-07	6.50E-09	3.44E-09		>1.25E-06	9.14E-07	2.75E-07	8.39E-07

[0086] The compound of the present invention is shown to have high potency *in vitro*, when compared against reference compounds. This demonstrates that the compound according to the present invention exhibits high cytotoxicity towards cancer cells and are useful in the treatment of cancer.

Example 4. MTD and MTMD determination

[0087] Female CD-1 or Athymic Nude-Foxn1 nu/nu mice (Envigo) were utilized for all experiments. Animals (N=10/cage) were housed in individually ventilated cages (Sealsafe Plus®, Techniplast S.P.A.), on a 12-hour light-dark cycle at 21-23 °C and 40-60% humidity. Mice were allowed free access to irradiated standard rodent diet (Tecklad 2914C) and sterilized water. Animals were acclimated for five days prior to being individually tattoo-identified. Animal protocols were reviewed and approved according to the regional Institutional Animal Care and Use Committees.

[0088] Mice were randomly allocated into experimental groups and intravenously administered, once for the MTD (Maximum Tolerated Dose) determination or one administration a week during three consecutive weeks, for the MTMD (Maximum Tolerated Multiple Dose) determination study. The animals were administered with white formulation or with compound dissolved in the experimental formulation at different concentrations. The volume administered was always 10 mL/kg. Once administered, animals were monitored for clinical signs of systemic toxicity, changes in body weight and mortality up to 14 days after the administration.

[0089] MTD results are summarized in **Table 3**

Table 3

Compound	Route / Schedule	Doses (mg/Kg)	MTD (mg/kg)
4-S	iv / SD	0.00, 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 5.00	1.0
Comp C		0.00, 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 5.00	3.0

[0090] MTMD results are summarized in **Table 4**

Table 4

Compound	Route / Schedule	Doses (mg/Kg)	MTMD (mg/kg)
4-S	iv / Q7dx3	0.00, 0.50, 0.75, 1.00, 1.25	1.25
Comp C		0.0, 1.0, 1.5, 2.0, 3.0, 4.0	3.0
iv, intravenously Q7dx3, three cumulated doses administered in a weekly basis.			

10 Examples 5-6. *In vivo* xenografts

[0091] Female athymic nu/nu mice (Harlan Laboratories Models, S.L. Barcelona, Spain or Envigo, Spain) were utilized for all experiments. Animal were housed in individually ventilated cages Sealsafe® Plus, Techniplast S.P.A.), up to ten per cage on a 12-hour light-dark cycle at 21-23 °C and 40-60 % humidity. Mice were allowed free access to irradiated standard rodent diet (Tecklad 2914C) and sterilized water. Animals were acclimated for at least 5 days prior to tumor implantation with a tumor cell suspension.

CELL LINES

Name	Nº ATCC	Nº ECCC*	Species	Tissue	Characteristics
HT1080	CCL-121	-	human	connective	Fibrosarcoma
MDA-MB-231	HTB-26	-	human	breast	Breast adenocarcinoma
H460	HTB-177	-	human	lung, pleural effusion	NSCLC
A2780	-	93112519	human	ovarian	Ovarian carcinoma
HGC27	-	94042256	human	gastric	Gastric carcinoma
H526	CRL-5811	-	human	lung	SCLC
H82	HTB-175	-	human	lung	SCLC
PC3	CLR-1435	-	human	prostate; derived from metastatic site: bone	Prostatic adenocarcinoma
DU145	HTB-81		human	prostate; derived from metastatic site: brain	Prostatic carcinoma
22Rv1	CRL-2505		human	prostate	Prostatic carcinoma

* European Collection of Cell Cultures

[0092] HT1080 cells were maintained *in vitro* at 37 °C with 5% CO₂ in Minimum Essential Medium Eagle (MEME) (Sigma-Aldrich, Co). Each animal was orthotopically implanted into gastrocnemius muscle by an intramuscular injection using a 26G needle and a 1 cc syringe at 4-6 weeks of age, with 10x10⁶ HT1080 cells, suspended in serum free medium, without antibiotics.

[0093] MDA-MB-231 cells were maintained *in vitro* at 37 °C with 5% CO₂ in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Co). Culture cells were passaged every 3 to 5 days upon reaching confluence. Each animal was subcutaneously implanted (on the right flank using 26G needle and a 1 cc syringe) at 4-6 weeks of age with 7.5x10⁶ MDA-MB-231 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0094] H460 cells were maintained *in vitro* at 37 °C with 5% CO₂ in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Co). Culture cells were passaged every 3 to 5 days upon reaching confluence. Each animal was subcutaneously implanted (on the right flank using 26G needle and a 1 cc syringe) at 4-6 weeks of age with 5x10⁶ H460 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0095] A2780 cells were maintained *in vitro* at 37 °C with 5% CO₂ in RPMI-1640 (Sigma-Aldrich, Co). Culture cells were passaged every 3 to 5 days upon reaching confluence. Each animal was subcutaneously implanted (on the right flank using 26G needle and a 1 cc syringe) at 4-6 weeks of age with 10x10⁶ A2780 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0096] HGC27 cells were maintained *in vitro* at 37 °C with 5% CO₂ in Iscove's Modified Dulbecco's Medium (Sigma Aldrich, Co). Culture cells were passage every 3 to 5 days on reaching confluence. Each animal was subcutaneously implanted (on the right flank using 26G needle and a 1 cc syringe) at 4-6 weeks of age with 5x10⁶ HGC-27 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® (Corning Incorporated Life Sciences), 50% medium without serum or antibiotics.

[0097] H526 cells were maintained *in vitro* at 37 °C with 5% CO₂ in RPMI-1640 Medium (Sigma-Aldrich, Co). H526 cells were grown as a suspension and maintained by addition of fresh medium, as the cell density increases, every 2 to 3 days. Every week, culture was reestablished by centrifugation of the suspension with subsequent resuspension in fresh medium at a concentration of 1x10⁵ cell/mL. Each animal was subcutaneously implanted (on the right flank using 26G needle and a 1 cc syringe) at 4-6 weeks of age with 5x10⁶ H526 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0098] H82 cells were maintained *in vitro* at 37 °C with 5% CO₂ in RPMI-1640 Medium (Sigma-Aldrich, Co). H82 cells were grown as a suspension and maintained by addition of fresh medium, as the cell density increases, every 2 to 3 days. Every week, culture was reestablished by centrifugation of the suspension with subsequent resuspension in fresh medium at a concentration of 1x10⁵ cell/ml. Animals were subcutaneously implanted (on the right flank using 26G needle and a 1 cc syringe) at 4-6 weeks of age with 5x10⁶ H82 cells, suspended in 0.05 mL of a solution consisting of 50% Matrigel® (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0099] PC3 cells were maintained *in vitro* at 37 °C with 5 % CO₂ in RPMI-1640 Medium (Sigma-Aldrich, Co). Culture cells were passaged every 3 to 5 days upon reaching confluence. Each female athymic mice was subcutaneously implanted (on the right flank using a 26G needle and a 1 cc syringe) at 4-6 weeks of age with 3x10⁶ PC3 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® Matrix (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0100] DU-145 cells were maintained *in vitro* at 37 °C with 5 % CO₂ in RPMI-1640 Medium (Sigma-Aldrich, Co). Culture cells were passaged every 3 to 5 days upon reaching confluence. Each male athymic mice was subcutaneously implanted (on the right flank using a 26G needle and a 1 cc syringe) at 4-6 weeks of age with 5x10⁶ DU-145 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® Matrix (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0101] 22Rv1 cells were maintained *in vitro* at 37 °C with 5 % CO₂ in RPMI-1640 Medium (Sigma-Aldrich, Co). Culture cells were passage every 3 to 5 days upon reaching confluence. Each male athymic mice was subcutaneously implanted (on the right flank using 26G needle and a 1 cc syringe) at 4-6 weeks of age with 5x10⁶ 22Rv1 cells suspended in 0.05 mL of a solution consisting of 50% Matrigel® Matrix (Corning Incorporated Life Sciences) and 50% medium without serum or antibiotics.

[0102] Treatment tolerability was assessed by monitoring body weight evolution, clinical signs of systemic toxicity, as well as evidences of local damage in the injection site.

[0103] In xenograft studies with HT1080 cell line:

- Total diameter (tumor + leg) measurements were determined by using digital caliper (Fowler Sylvac, S235PAT). This total diameter and animal body weights were measured 2-3 times per week starting from the first day of treatment (day 0).
- When total diameter reached a length of about 7.0-8.0 mm, mice were randomly allocated into the treatments and control groups (N = 8-10/group) based on body weight and tumor measurements by using NewLab Oncology Software (version 2.25.06.00).
- Comparison of the median total diameter (tumor + leg) in the treatment groups to the median total diameter (tumor + leg) in the control group was used for evaluation of the antitumoral efficacy.
- Animals were euthanized when their total leg diameter reached ca. 18 mm.

[0104] In xenograft studies with other cell lines:

- Tumor volume was calculated using the equation (a·b²)/2, where a: length (longest diameter) and b: width (shortest diameter) were measured in mm by using digital caliper (Fowler Sylvac, S235PAT). Tumor dimensions and body weights were recorded 2-3 times per week starting from the first day of treatment.
- When tumors reached ca. 150-250 mm³, tumor bearing animals (N = 8-10/group) were randomly allocated into the treatment groups, based on body weight and tumor measurements by using NewLab Oncology Software (version 2.25.06.00).

- Comparison between median tumor volume of treated groups and control group was used for evaluation of the antitumoral efficacy.
- Animals were euthanized when their tumors reached ca. 2000 mm³ and/or severe necrosis was seen.

5

[0105] Treatments producing >20 % lethality and/or 20% net body weight loss were considered toxic.

[0106] Tables and figures summarize the data obtained from complete experimental groups, i.e. those groups keeping the initial number of animals, n = 8-10. However, once the first animal is sacrificed due to a tumor length > 18 mm or a tumor size > 2000 mm³, the experimental group will be considered incomplete. Therefore, data generated subsequently to the sacrifice day and onwards will not be presented (i.e. neither in tables nor in the figures).

10

Example 5. *In vivo* studies to determine the effect of **4-S** in several xenograft models

[0107] **4-S** and compound **C** were provided in the form of freeze-dried vials of lyophilized product. Each vial was reconstituted with water for infusion to a concentration of 0.5 mg/mL. Further dilutions were made with 5% dextrose solution for injection to the dosing formulation concentration. The administered doses of **4-S** and compound **C** were 1.25 mg/kg and 3.0 mg/kg, respectively.

[0108] Placebo was provided in the form of lyophilised cake containing 100 mg Sucrose + Potassium dihydrogen phosphate 6.8 mg + Phosphoric acid q.s. pH 3.8-4.5 which was reconstituted with water for infusion.

[0109] In these experiments, **4-S** and Compound **C**, as well as placebo, were intravenously administered once per week for 3 consecutive weeks, on Days 0, 7 and 14, whenever it was possible.

20

Example 5a. *In vivo* studies to determine the effect of **4-S** in human fibrosarcoma xenografts.

[0110] The aim of this study was to compare the antitumoral activity of **4-S** with the antitumoral activity of compound **C** by using a xenograft model of human sarcoma.

[0111] The tumor model used in this study was HT1080 cell line.

[0112] **Table 5** reports the total diameter (tumor + leg) evaluation of HT1080 tumors in mice treated with placebo, compound **C**, and **4-S**. These results are also showed in Figure 1.

30

Table 5

Days	Total diameter (tumor + leg) (mm)		
	Control	Compound C	4-S
0.0	7.5	7.5	7.5
2.0	9.4	8.8	7.7
5.0	11.4	9.0	8.3
7.0	12.1	9.6	8.8
9.0	13.2	10.2	8.4
12.0	14.5	10.2	8.4
14.0	15.2	11.2	9.6
16.0	15.9	12.4	10.0
19.0	18.0	13.3	10.4
21.0		15.2	12.1
23.0		18.0	12.7
27.0			13.5
30.0			15.6
33.0			18.0

55

Example 5b. *In vivo* studies to determine the effect of **4-S** in human breast xenografts.

[0113] The aim of this study was to compare the antitumoral activity of **4-S** with the antitumoral activity of compound **C** by using a xenograft model of human breast cancer.

5 [0114] The tumor model used in this study was MDA-MB-231 cell line.

[0115] **Table 6** reports the median tumor volume evaluation of MDA-MB-231 tumors in mice treated with placebo, compound **C** and **4-S**. These results are also showed in Figure 2.

10 **Table 6**

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
0.0	149.4	149.4	150.6
2.0	240.0	217.1	197.3
5.0	325.1	281.3	250.9
7.0	407.8	338.6	265.0
9.0	514.8	385.1	272.5
12.0	648.1	400.4	270.6
14.0	799.0	436.9	281.3
16.0	1002.5	585.7	293.6
19.0	1233.9	774.7	322.1
21.0	1539.1	965.9	324.4
23.0	2006.5	1215.2	326.6
26.0	2027.7	1503.2	398.8
28.0		1785.3	501.8
30.0		2037.1	654.8
33.0			856.7
35.0			1147.1
37.0			1635.9

40 **Example 5c.** *In vivo* studies to determine the effect of **4-S** in human lung tumor xenografts.

[0116] The aim of this study was to compare the antitumoral activity of **4-S** with the antitumoral activity of compound **C** by using three different xenograft models of human lung cancer. These models correspond to non-small cell lung cancer (H-460 cell line) and to small cell lung cancer (H526 and H82 cell lines).

45 [0117] **Table 7** reports the median tumor volume evaluation of H460 tumors in mice treated with placebo, compound **C** and **4-S**. These results are also showed in Figure 3.

50 **Table 7**

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
0.0	187.4	186.1	185.9
2.0	577.5	395.4	310.9
5.0	1352.0	665.9	634.6
7.0	1642.9	929.5	959.1
9.0	2025.0	1063.7	1064.9

(continued)

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
12.0		1436.5	1421.0
14.0		2025.0	1845.5
16.0		2025.0	2025.0

[0118] **Table 8** reports the median tumor volume evaluation of H526 tumors in mice treated with placebo, compound **C** and **4-S**. These results are also showed in Figure 4.

Table 8

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
0.0	217.2	217.9	211.8
2.0	410.7	262.4	279.0
4.0	778.5	108.3	98.8
7.0	1083.2	129.8	56.7
9.0	1371.0	85.9	62.5
11.0	1782.0	52.3	32.0
14.0	2025.0	54.1	18.0
16.0		47.3	32.0
21.0		4.0	4.0
28.0		4.0	4.0
35.0		4.0	4.0
42.0		62.5	4.0
49.0		53.5	4.0

[0119] **Table 9** reports the median tumor volume evaluation of H82 tumors in mice treated with placebo, compound **C** and **4-S**. These results are also showed in Figure 5.

Table 9

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
0.0	171.6	170.5	168.3
2.0	439.4	265.3	215.2
5.0	1024.7	488.7	253.6
7.0	1422.0	760.0	341.4
9.0	1923.8	899.5	349.4
12.0	2025.0	1038.5	436.4
14.0		1213.4	516.0
16.0		1256.4	521.8
19.0		1741.5	560.9

(continued)

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
21.0		1878.8	627.7
23.0		2057.0	690.9
26.0			953.4
28.0			847.1
30.0			1067.5
33.0			1200.6
35.0			1257.7
37.0			1497.7
41.0			2014.2

20 **Example 5d.** *In vivo* studies to determine the effect of **4-S** in human ovarian tumor xenografts.

[0120] The aim of this study was to compare the antitumoral activity of **4-S** with the antitumoral activity of compound **C** by using a xenograft model of human ovarian cancer.

[0121] The tumor model used in this study was A2780.

25 [0122] **Table 10** reports the volume evaluation of A2780 tumors in mice treated with placebo, compound **C** and **4-S**. These results are also showed in Figure 6.

Table 10

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
0.0	169.5	169.6	168.3
2.0	317.5	206.3	150.6
5.0	758.9	372.7	175.9
7.0	1351.9	607.6	317.7
9.0	1675.8	696.2	281.9
12.0	2025.0	855.6	372.1
14.0		1293.9	709.2
16.0		1683.5	870.9
19.0		2137.5	1235.4
21.0			1453.3
23.0			1666.0
26.0			2025.0

50 **Example 5e.** *In vivo* studies to determine the effect of **4-S** in human gastric tumor xenografts.

[0123] The aim of this study was to compare the antitumoral activity of **4-S** with the antitumoral activity of Compound **C** by using a xenograft model of human gastric cancer.

[0124] The tumor model used in this study was HGC27.

55 [0125] **Table 11** reports tumor volume growth of HGC27 tumors in mice treated with placebo, compound **C** and **4-S**. These results are also showed in Figure 7.

Table 11

Days	Median Tumor Volume (mm ³)		
	Control	Compound C	4-S
0.0	200.7	195.0	194.8
2.0	429.0	391.0	358.6
5.0	835.5	578.6	515.3
7.0	1256.5	708.2	589.2
9.0	1602.2	937.7	779.4
12.0	2040.7	1169.5	980.8
14.0		1496.8	1153.3
16.0		1690.6	1346.2
19.0		2004.0	1643.4
21.0			2004.7

Example 6 (for reference).

[0126] *In vivo* studies to determine the effect of **4-S** in human prostate xenografts. The aim of this study was to compare the antitumoral activity of **4-S** by using three different xenograft models of human prostate cancer. These models correspond to PC-3, DU-145 and 22Rv1 cell lines.

[0127] Compound **4-S** was provided in the form of freeze-dried vials of lyophilized product. Each vial was reconstituted with sterile water for injection to a concentration of 0.5 mg/mL. Further dilutions were made with 5% dextrose solution for injection to the dosing formulation concentration. The administered dose of **4-S** varied depending on the study, being 1.25 mg/Kg when the tumor model was PC-3, 1.00 mg/Kg when the tumor model was DU-145 and 0.75 mg/Kg when the tumor model was 22Rv1, respectively.

[0128] Placebo was provided in the form of lyophilised cake containing 100 mg Sucrose + Potassium dihydrogen phosphate 6.8 mg + Phosphoric acid q.s. pH 3.8-4.5 which was reconstituted with water for infusion.

[0129] In these experiments, **4-S**, as well as placebo were intravenously administered once per week for 3 consecutive weeks, on Days 0, 7 and 14, whenever it was possible.

[0130] Table 12 reports the median tumor volume evaluation of PC-3 tumors in mice treated with placebo and **4-S**. These results are also showed in Figure 8.

Table 12

Median Tumor Volume (mm ³)		
Days	Control	4-S
0	140.5	141.3
2	178.6	130.7
4	233.1	147.6
7	284.6	157.7
9	331.7	200.9
11	433.7	192.8
14	500.4	210.8
16	570.8	255.5
18	680.3	261.1
21	850.1	282.4
23	928.5	382.2

(continued)

Median Tumor Volume (mm ³)		
Days	Control	4-S
25	915.7	451.6
28	1187.5	611.1
30	1270.1	762.3
32	1327.1	821.6
35	1373.6	1045.6

5 [0131] **Table 13** reports the median tumor volume evaluation of DU-145 tumors in mice treated with placebo and **4-S**. These results are also showed in Figure 9.

10 **Table 13**

Median Tumor Volume (mm ³)		
Days	Control	4-S
0	127.4	126.2
3	180.9	102.4
5	248.8	119.5
7	320.4	149.5
10	384.6	216.8
12	441.0	181.4
14	519.6	237.7
17	601.0	204.4
19	660.8	210.9
24	740.7	300.0
26	798.6	378.4
28		587.0
31		650.3

15 20 25 30 35 40 [0132] **Table 14** reports the median tumor volume evaluation of 22Rv1 tumors in mice treated with placebo and **4-S**. These results are also showed in Figure 10.

45 **Table 14**

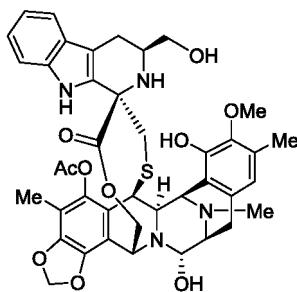
Median Tumor Volume (mm ³)		
Days	Control	4-S
0	174.6	173.6
3	307.2	70.3
5	511.5	63.1
7	739.1	76.7
10	955.2	49.1
12	1286.1	59.8
14	1385.8	74.9

(continued)

Median Tumor Volume (mm ³)		
Days	Control	4-S
17	1791.1	55.1
19	2025.0	64.9
24		138.4
26		186.9
28		242.0
31		392.5
33		561.8
35		799.3
38		1107.0
40		1426.4
42		1685.5
45		2025.0

Claims

1. A compound of formula I, or a pharmaceutically acceptable salt or ester thereof:



Formula I.

2. The compound according to claim 1, wherein the salt is selected from hydrochloride, hydrobromide, hydroiodide, sulfate, nitrate, phosphate, acetate, trifluoroacetate, maleate, fumarate, citrate, oxalate, succinate, tartrate, malate, mandelate, methanesulfonate, *p*-toluenesulfonate, sodium, potassium, calcium and ammonium salts; ethylenediamine, ethanolamine, *N,N*-dialkylenglycolamine, triethanolamine and basic aminoacids salts.
3. A pharmaceutical composition comprising a compound according to any one of claims 1 to 2 or a pharmaceutically acceptable salt or ester thereof and a pharmaceutically acceptable carrier.
4. A dosage form comprising a pharmaceutical composition according to claim 3.
5. A compound according to any one of claims 1 to 2, or a pharmaceutically acceptable salt or ester thereof, a composition according to claim 3, or a dosage form according to claim 4, for use as a medicament.
6. A compound according to any one of claims 1 to 2, or a pharmaceutically acceptable salt or ester thereof, a composition according to claim 3, or a dosage form according to claim 4, for use in the treatment of cancer.
7. The compound, composition, or dosage form according to claim 6, wherein the cancer is selected from lung cancer including non-small cell lung cancer and small cell lung cancer, colon cancer, colorectal cancer, breast cancer,

pancreas cancer, sarcoma, ovarian cancer, and gastric cancer.

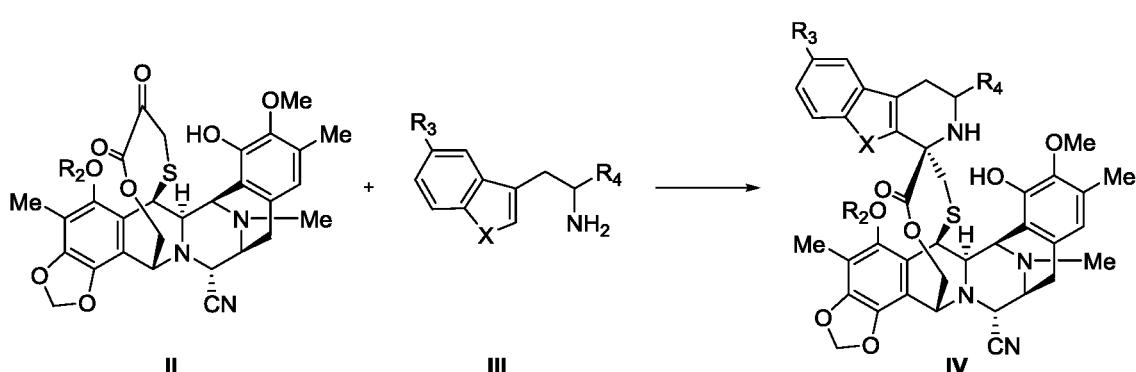
8. The compound, composition or dosage form according to claim 7, wherein the cancer is selected from lung cancer including non-small cell lung cancer and small cell lung cancer, breast cancer, pancreas cancer and colorectal cancer.

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9. A process for obtaining a compound of formula I as defined in claim 1 or a pharmaceutically acceptable salt or ester thereof:

comprising the step of reacting a compound of formula II with a compound of formula III to give a compound of formula IV:

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wherein:

X is -NH-;
R₂ is acetyl;
R₃ is hydrogen;
R₄ is -CH₂OH.

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10. The process according to claim 9, comprising the further step of replacing the cyano group in the compound of formula IV with a hydroxy group to give a compound of formula I.

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11. A kit comprising a therapeutically effective amount of a compound according to any one of claims 1 to 2 and a pharmaceutically acceptable carrier.

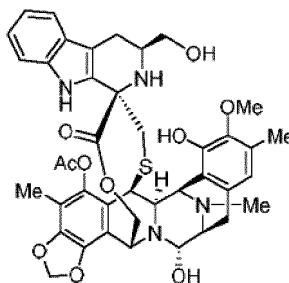
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12. The kit according to claim 11 further comprising instructions for use of the compound in the treatment of cancer, and more preferably a cancer selected from lung cancer, including non-small cell lung cancer and small cell lung cancer, colon cancer, colorectal cancer, breast cancer, pancreas cancer, sarcoma, ovarian cancer, and gastric cancer.

Patentansprüche

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1. Verbindung der Formel I, oder ein pharmazeutisch verträgliches/-r Salz oder Ester davon:



Formel I.

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2. Verbindung nach Anspruch 1, wobei das Salz aus Hydrochlorid, Hydrobromid, Hydrojodid, Sulfat, Nitrat, Phosphat, Acetat, Trifluoracetat, Maleat, Fumarat, Citrat, Oxalat, Succinat, Tartrat, Malat, Mandelat, Methansulfonat, *p*-Toluenulfonat; Natrium-, Kalium-, Kalzium- und Ammoniumsalzen; Ethyldiamin-, Ethanolamin-, *N,N*-Dialkylenethanolamin-, Triethanolaminsalzen und Salzen basischer Aminosäuren ausgewählt ist.

5 3. Pharmazeutische Zusammensetzung, umfassend eine Verbindung nach einem der Ansprüche 1 bis 2 oder ein/-en pharmazeutisch verträgliches/-n Salz oder Ester davon und einen pharmazeutisch verträglichen Träger.

10 4. Darreichungsform, umfassend eine pharmazeutische Zusammensetzung nach Anspruch 3.

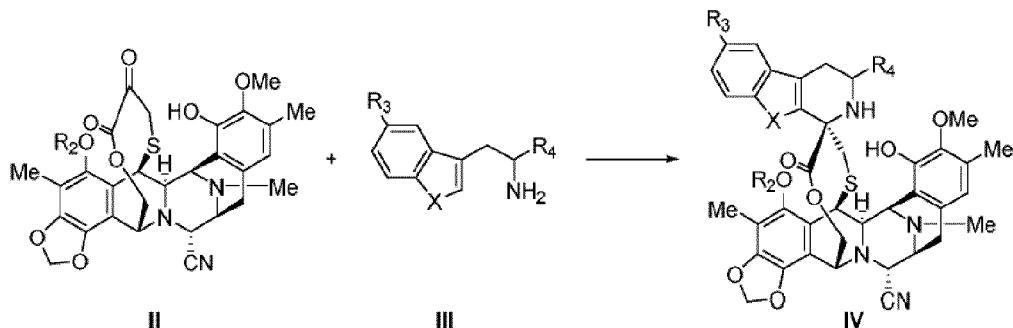
15 5. Verbindung nach einem der Ansprüche 1 bis 2, oder ein pharmazeutisch verträgliches/-r Salz oder Ester davon, eine Zusammensetzung nach Anspruch 3, oder eine Darreichungsform nach Anspruch 4, zur Verwendung als Medikament.

20 6. Verbindung nach einem der Ansprüche 1 bis 2, oder ein pharmazeutisch verträgliches Salz/-r oder Ester davon, eine Zusammensetzung nach Anspruch 3, oder eine Darreichungsform nach Anspruch 4, zur Verwendung in der Behandlung von Krebs.

25 7. Verbindung, Zusammensetzung oder Darreichungsform nach Anspruch 6, wobei der Krebs aus Lungenkrebs einschließlich nicht kleinzelligen Lungenkrebses und kleinzelligen Lungenkrebses, Kolonkrebs, Kolorektalkrebs, Brustkrebs, Bauchspeicheldrüsenkrebs, Sarkom, Eierstockkrebs und Magenkrebs ausgewählt ist.

30 8. Verbindung, Zusammensetzung oder Darreichungsform nach Anspruch 7, wobei der Krebs aus Lungenkrebs einschließlich nicht kleinzelligen Lungenkrebses und kleinzelligen Lungenkrebses, Brustkrebs, Bauchspeicheldrüsenkrebs und Kolorektalkrebs ausgewählt ist.

9. Verfahren zur Gewinnung einer Verbindung der Formel I gemäß Definition in Anspruch 1 oder eines pharmazeutisch verträglichen Salzes oder Esters davon:
umfassend den Schritt des Umsetzens einer Verbindung der Formel II mit einer Verbindung der Formel III unter Erhalt einer Verbindung der Formel IV:



wobei:

X -NH- ist;
R₂ Acetyl ist;
R₃ Wasserstoff ist;
R₄ -CH₂OH ist.

10. Verfahren nach Anspruch 9, umfassend den weiteren Schritt des Ersetzens der Cyanogruppe in der Verbindung der Formel IV durch eine Hydroxygruppe unter Erhalt einer Verbindung der Formel I.

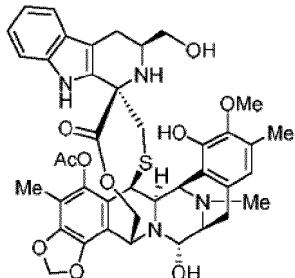
11. Kit, umfassend eine therapeutisch wirksame Menge einer Verbindung nach einem der Ansprüche 1 bis 2 und einen pharmazeutisch verträglichen Träger.

12. Kit nach Anspruch 11, weiterhin umfassend Anweisungen zur Verwendung der Verbindung in der Behandlung von Krebs, und stärker bevorzugt von einem Krebs, der aus Lungenkrebs, einschließlich nicht kleinzelligen Lungen-

krebses und kleinzelligen Lungenkrebses, Kolonkrebs, Kolorektalkrebs, Brustkrebs, Bauchspeicheldrüsenkrebs, Sarkom, Eierstockkrebs und Magenkrebs ausgewählt ist.

5 **Revendications**

1. Composé de formule I, ou sel ou ester pharmaceutiquement acceptable de celui-ci :



Formule I.

10 2. Composé selon la revendication 1, dans lequel le sel est sélectionné parmi le chlorhydrate, le bromhydrate, l'iodhydrate, le sulfate, le nitrate, le phosphate, l'acétate, le trifluoroacétate, le maléate, le fumarate, le citrate, l'oxalate, le succinate, le tartrate, le malate, le mandélate, le méthanesulfonate, le *p*-toluènesulfonate ; les sels de sodium, de potassium, de calcium et d'ammonium ; les sels d'éthylènediamine, d'éthanolamine, de la *N,N*-dialkylène-éthanolamine, de la triéthanolamine et les sels d'acides aminés basiques.

15 3. Composition pharmaceutique comprenant un composé selon l'une quelconque des revendications 1 à 2 ou un sel ou un ester pharmaceutiquement acceptable de celui-ci et un véhicule pharmaceutiquement acceptable.

20 4. Forme galénique comprenant une composition pharmaceutique selon la revendication 3.

25 5. Composé selon l'une quelconque des revendications 1 à 2, ou sel ou ester pharmaceutiquement acceptable de celui-ci, composition selon la revendication 3 ou forme galénique selon la revendication 4, pour une utilisation en tant que médicament.

30 6. Composé selon l'une quelconque des revendications 1 à 2, ou sel ou ester pharmaceutiquement acceptable de celui-ci, composition selon la revendication 3 ou forme galénique selon la revendication 4, pour une utilisation dans le traitement du cancer.

35 7. Composé, composition ou forme galénique selon la revendication 6, dans lequel le cancer est sélectionné parmi le cancer du poumon incluant le cancer du poumon non à petites cellules et le cancer du poumon à petites cellules, le cancer du côlon, le cancer colorectal, le cancer du sein, le cancer du pancréas, le sarcome, le cancer de l'ovaire et le cancer de l'estomac.

40 8. Composé, composition ou forme galénique selon la revendication 7, dans lequel le cancer est sélectionné parmi le cancer du poumon incluant le cancer du poumon non à petites cellules et le cancer du poumon à petites cellules, le cancer du sein, le cancer du pancréas et le cancer colorectal.

45 9. Procédé destiné à obtenir un composé de formule I tel que défini dans la revendication 1 ou un sel ou un ester pharmaceutiquement acceptable de celui-ci :
comportant l'étape consistant à faire réagir un composé de formule II avec un composé de formule III pour obtenir un composé de formule IV :

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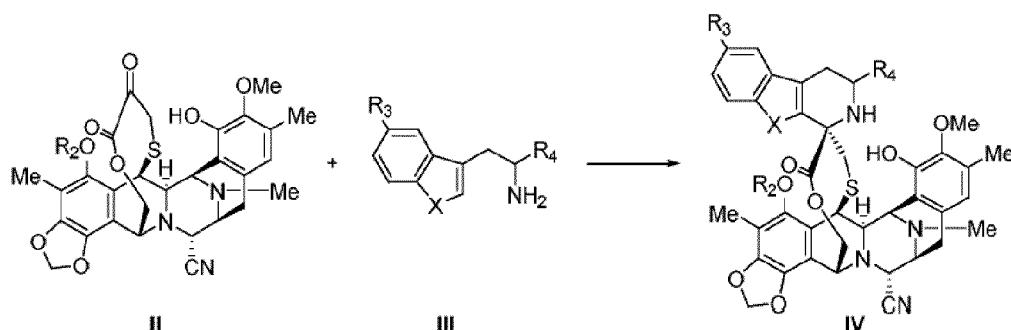
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dans lequel :

X est -NH- ;
 R₂ est l'acétyle ;
 R₃ est l'hydrogène ;
 R₄ est -CH₂OH.

10. Procédé selon la revendication 9, comprenant l'étape supplémentaire consistant à remplacer le groupe cyano dans le composé de formule **IV** avec un groupe hydroxy pour obtenir un composé de formule **I**.
11. Kit comprenant une quantité thérapeutiquement efficace d'un composé selon l'une quelconque des revendications 1 à 2 et d'un véhicule pharmaceutiquement acceptable.
12. Kit selon la revendication 11 comprenant en outre des instructions pour l'utilisation du composé dans le traitement du cancer, et plus préféablement un cancer sélectionné parmi le cancer du poumon, incluant le cancer du poumon non à petites cellules et le cancer du poumon à petites cellules, le cancer du côlon, le cancer colorectal, le cancer du sein, le cancer du pancréas, le sarcome, le cancer de l'ovaire et le cancer de l'estomac.

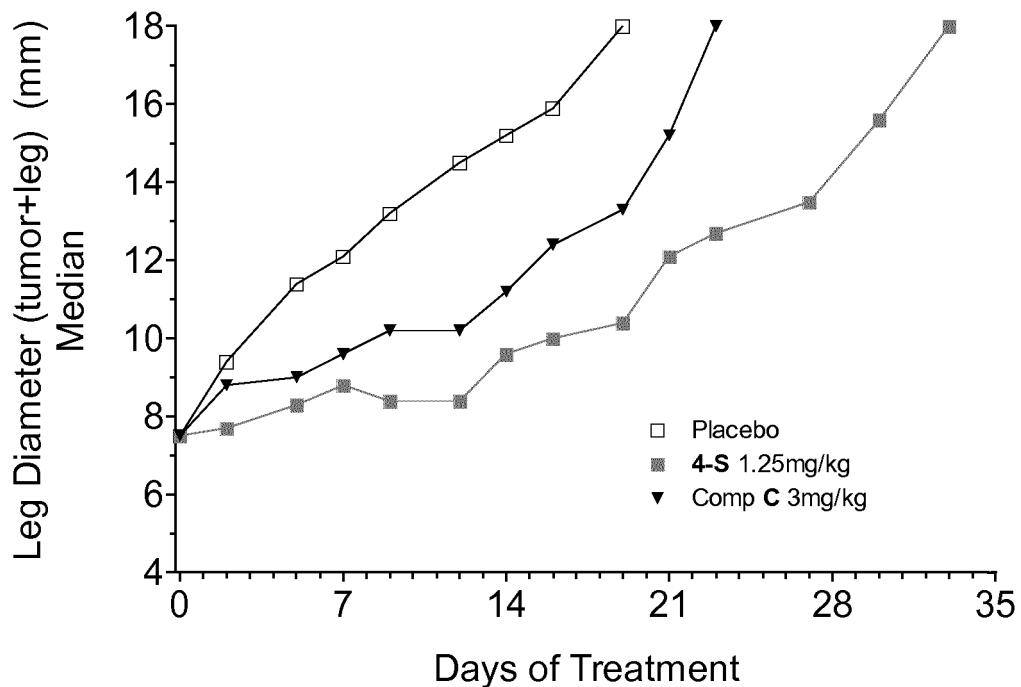


Figure 1

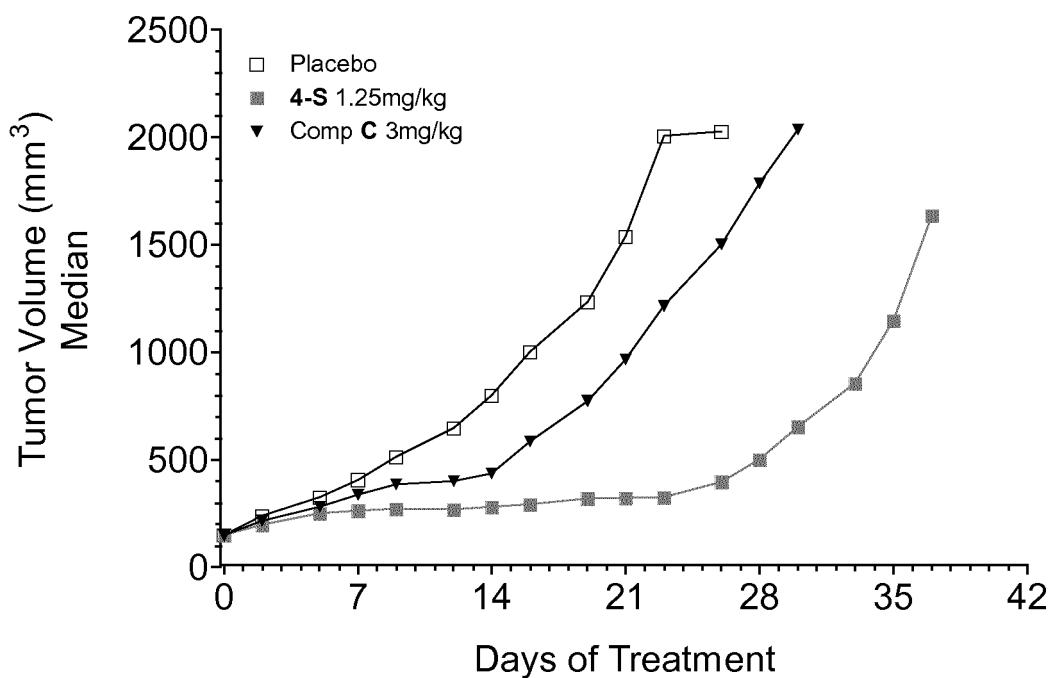


Figure 2

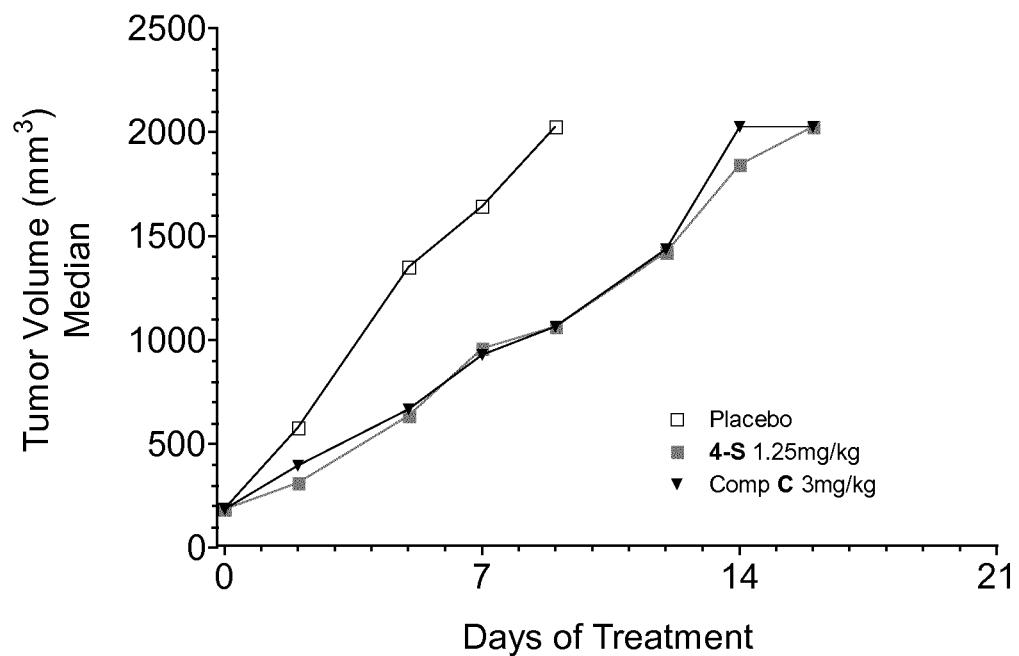


Figure 3

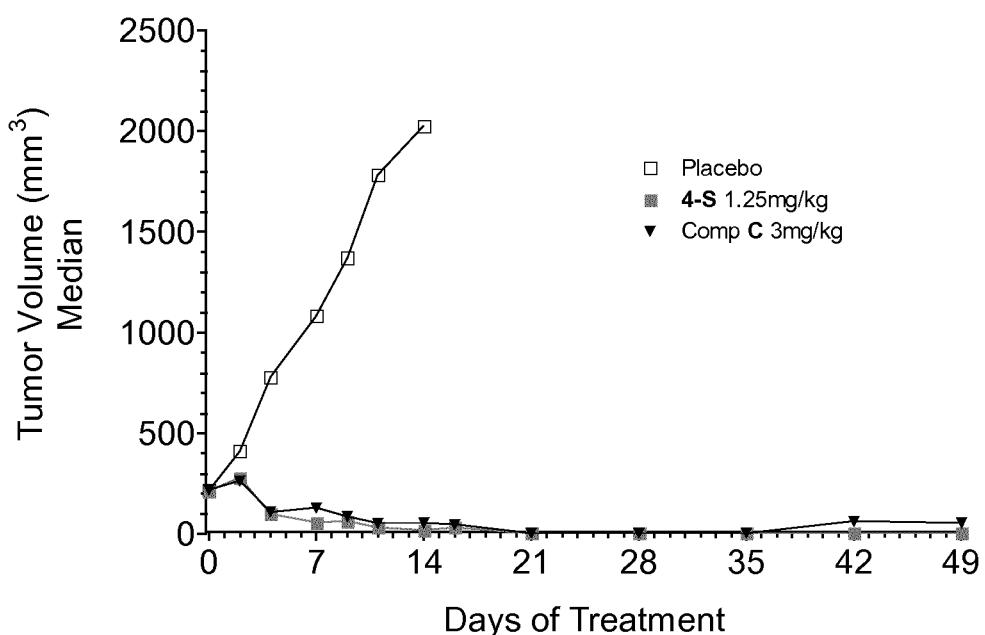


Figure 4

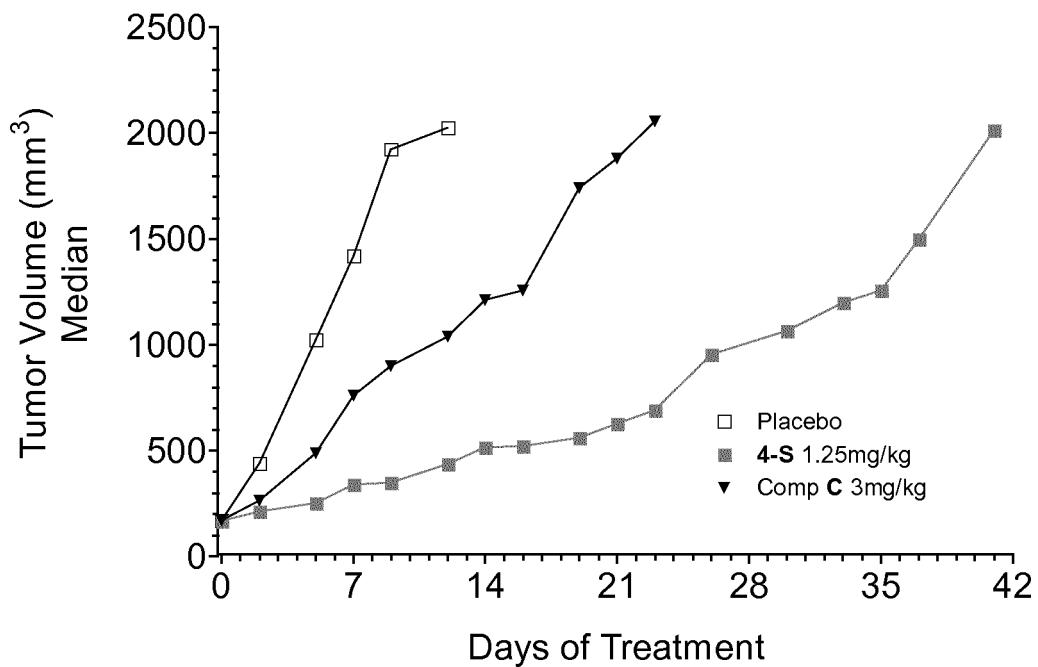


Figure 5

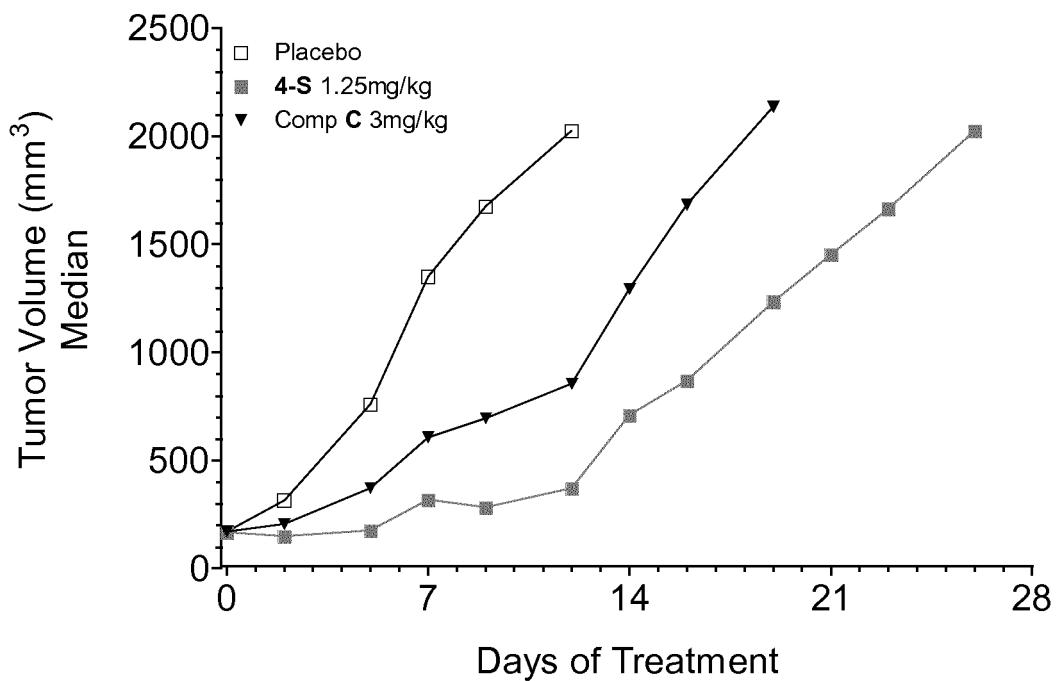


Figure 6

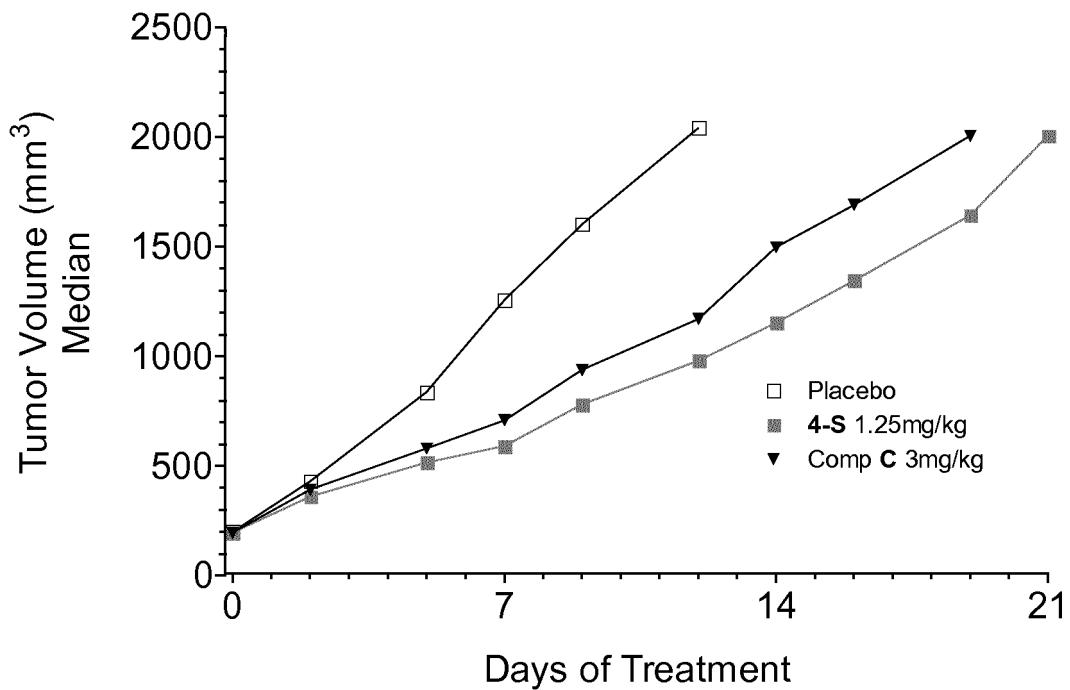


Figure 7

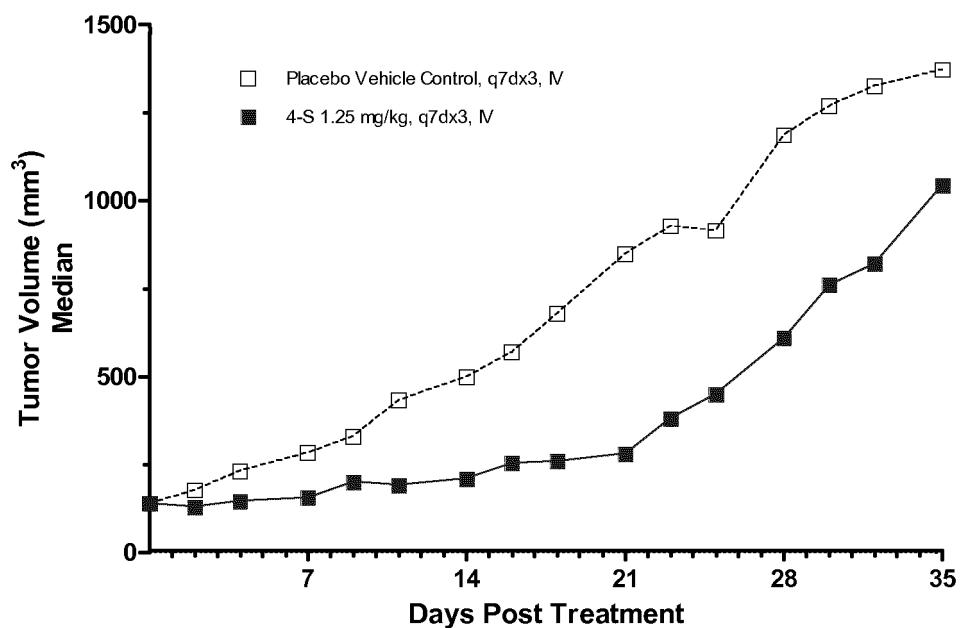


Figure 8

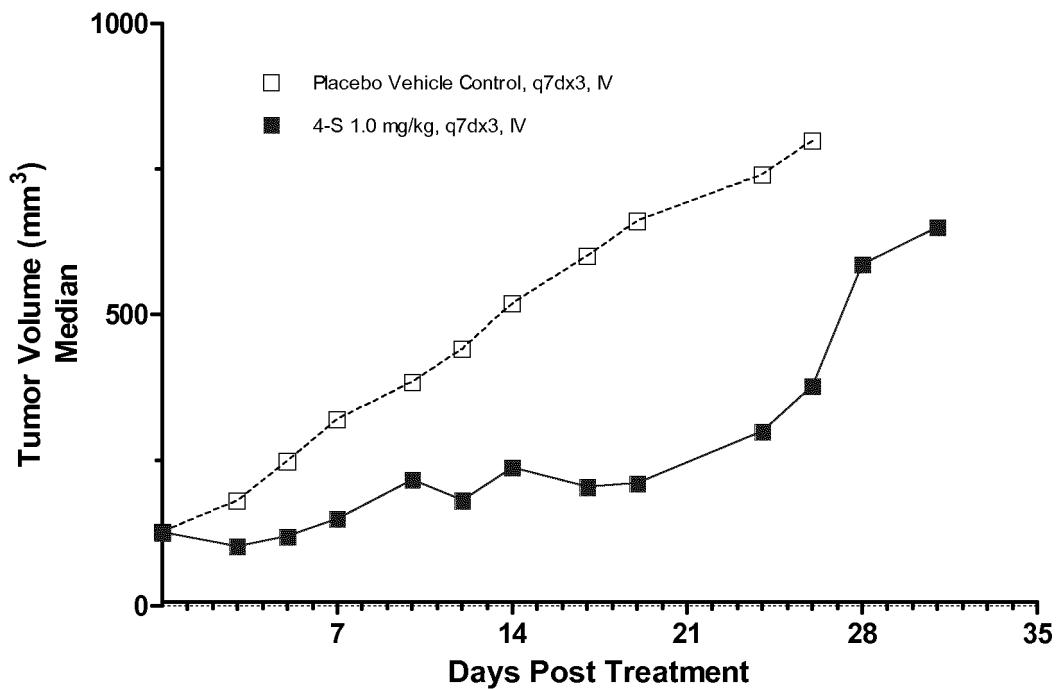


Figure 9

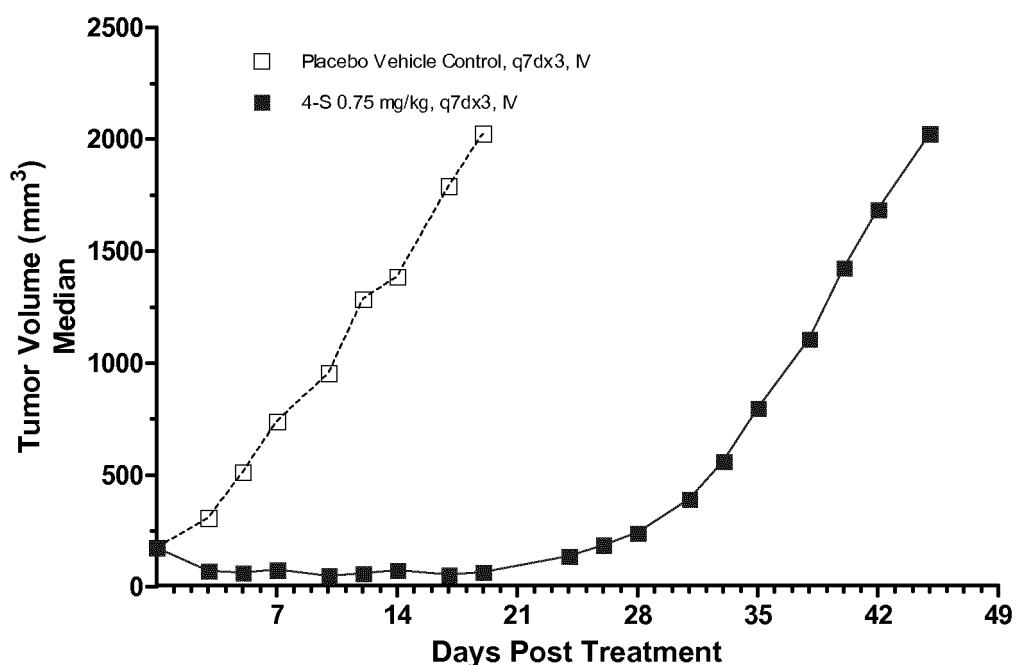


Figure 10

REFERENCES CITED IN THE DESCRIPTION

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