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(54) Title: NICLOSAMIDE AND ITS DERIVATIVES FOR USE IN THE TREATMENT OF SOLID TUMORS

(57) Abstract: The present invention relates to novel therapeutic uses of niclosamide for the treatment of cancer. In particular, a combination of niclosamide or one of its derivatives with an alkylating agent is provided for the treatment of solid tumors. Moreover, niclosamide or one of its derivatives can be used for the treatment of solid tumors characterized by underexpression of NFKBIA. Finally, the invention relates to diagnostic methods for determining whether treatment with niclosamide alone or in combination with an alkylating agent is suitable for a cancer patient.

NICLOSAMIDE AND ITS DERIVATIVES FOR USE IN THE TREATMENT OF SOLID TUMORS

5 The present invention relates to novel therapeutic uses of niclosamide for the treatment of cancer. In particular, a combination of niclosamide or one of its derivatives with an alkylating agent is provided for the treatment of solid tumors. Moreover, niclosamide or one of its derivatives can be used for the treatment of solid tumors characterized by underexpression of NFKBIA. Finally, the invention relates to diagnostic methods for determining whether
10 treatment with niclosamide alone or in combination with an alkylating agent is suitable for a cancer patient.

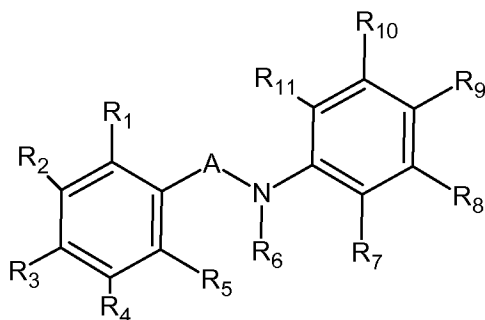
Background of the Invention

Chemotherapy has developed to an increasingly effective line of defense against cancer (1). In
15 glioblastoma (GBM), the alkylating agent temozolomide (TMZ) has become a standard, in combination with surgical resection and radiotherapy. There is nevertheless a considerable need to develop alternative treatment options, as GBM remains a fatal disease with a median overall survival of only 15 months (2, 3). For anticancer drug discovery, two major strategies are traditionally employed (4). One is the targeted approach where cancer-related molecules
20 and/or signaling cascades need to be exposed before specific compounds can be designed for distinct interference and inhibition. Alternatively, empirical screening of hundreds to thousands of compounds can be conducted to identify otherwise unpredictable antineoplastic effects. Both strategies, however, are burdened with high attrition rates during clinical translation (5, 6). This may in part be caused by the use of inept cellular model systems for
25 drug evaluation at early developmental stages (7), e.g. inter- and intra-patient tumor heterogeneity is rarely reflected within these systems. The present inventors have developed an *in vitro* cellular system that is closely mirroring GBM *in vivo* and is, thus, particular suitable to identify compounds and combinations of compounds that are likely to also provide GBM specific anticancer activity when used *in vivo*. Using this *in vitro* cellular system the
30 present inventors were able to identify compounds and compound combinations for the improved chemotherapy of glioblastoma.

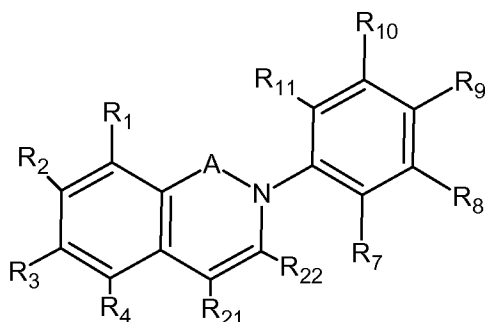
Summary of the Invention

In a first aspect the invention relates to a combination of a first cytostatic compound according to formula I, II or III

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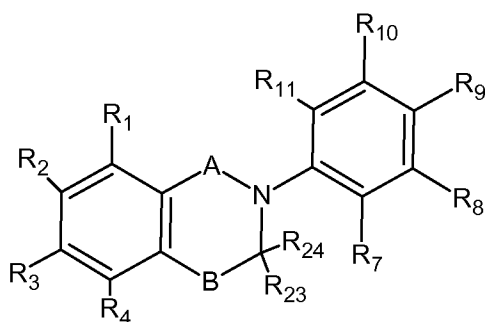


(I)



(II)

10



(III)

wherein

15

A is carbonyl, methylene, hydroxymethyl, alkoxymethyl, aminomethyl, oxime, hydrazone, arylhydrazone, or semicarbazone;

B if present is CR₂₅R₂₆, O, S or NR₂₇;

R₁, R₃, R₄, R₈, R₁₀, and R₁₁ are independently hydrogen; hydroxyl, alkoxy; halogen; or C₁ to

C₆ alkyl;

R₂ and R₇ are independently halogen, hydroxyl or hydrogen,

R₅ if present is hydroxyl, phosphate, hydrogen, halogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, alkoxy, alkylthio or amino;

5 R₆ if present is hydrogen or C₁ to C₆ alkyl;

R₉ is nitro, hydrogen, hydroxyl, amino, halogen, alkyl, alkenyl, alkynyl, or aryl; and

R₂₁, R₂₂, R₂₃, and R₂₄, if present are independently hydrogen, hydroxyl, halogen, alkyl, alkenyl, alkynyl, cycloalkyl or aryl;

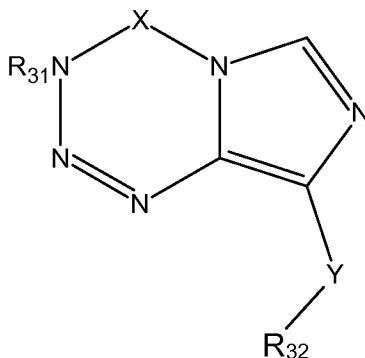
10 R₂₅ and R₂₆ if present are independently hydrogen, hydroxyl, halogen, alkyl, alkenyl, alkynyl, cycloalkyl or aryl; and

R₂₇ if present is hydrogen or C₁ to C₆ alkyl;

or salt thereof;

and an alkylating compound for use in treating of a solid tumor.

15 In an aspect of the invention, the alkylating compound has a structure according to formula IV



(IV)

20 wherein

X and Y are independently carbonyl, methylene, hydroxymethyl, alkoxyethyl, aminomethyl, oxime, hydrazone, arylhydrazone or semicarbazone,

R₃₁ is hydrogen, alkyl, alkoxy, alkenyl, alkynyl, cycloalkyl or aryl; and

R₃₂ is amino, hydrogen, hydroxyl or halogen

25 or salt thereof.

In a further aspect, the present invention relates to a pharmaceutical composition comprising a compound according to formula I, II or III, wherein A, B, R₁ to R₁₁ and R₂₁ to R₂₄ have a meaning as defined above, an alkylating agent and a pharmaceutically acceptable excipient

for use in treating of a solid tumor.

In yet a further aspect, the present invention relates to a compound according to formula I, II or III, wherein A, B, R₁ to R₁₁ and R₂₁ to R₂₄ have a meaning as defined above for use in
5 treating a solid tumor characterized by a decreased expression level NFKBIA.

In yet a further aspect, the present invention relates to a method for determining if therapy with the cytostatic compound according to formula I, II or III as defined supra is suitable for treating a patient with a solid tumor comprising the steps of

- 10 a) determining the expression level of NFKBIA in a sample of tumor cells or tumor tissue of the patient;
- b) comparing the determined expression level with a reference value;
- c) determining if the therapy with niclosamide is suitable for the patient based on the result of the comparison of step b), wherein underexpression or a deletion of NFKBIA
15 indicates that the combination therapy is suitable for the patient.

In yet another aspect, the present invention relates to a method for determining the molar ratio of niclosamide to temozolomide to be administered to a patient with a solid tumor comprising the steps of

- 20 a) determining the expression level of NFKBIA in a sample of tumor cells or tumor tissue of the patient;
- b) comparing the determined expression level with a reference value;
- c) determining the molar ratio of niclosamide to temozolomide based on the result of the comparison of step b), wherein
- 25 (i) an expression above the reference value indicates that the molar ratio shall be below 40 % niclosamide; and
- (ii) an expression level below the reference value indicates that the molar ratio shall be larger than or equal to 40 % niclosamide.

30 **Detailed Description of the Invention**

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H.G.W, Nagel, B. and Klbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

5 Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

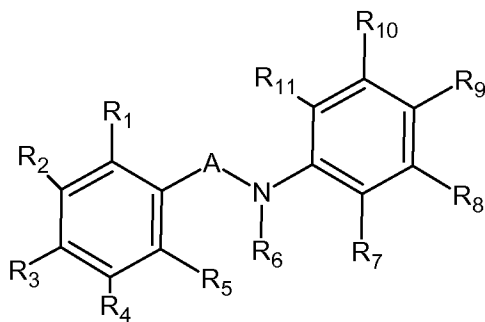
10 Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

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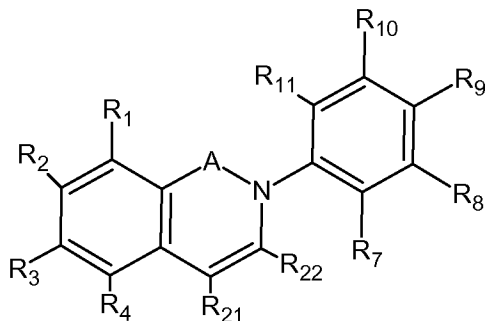
In order to identify novel compounds suitable for the treatment of solid tumors, in particular glioblastoma, primary human GBM cells known to retain patient- and disease-specific traits in vitro (pGBMs) (8-10), were applied in an empirical screening approach. Drug discovery and validation was conducted on a portfolio of pGBMs representing multiple facets of the disease. Three independent human non-malignant neural cell populations (hnNCs) as well as five commonly used glioma cell lines served as control for the experiments. The small molecule niclosamide emerged from these studies as pGBM-selective, pro-apoptotic, and antiproliferative compound. Niclosamide's pleiotropic mode of action effectively depleted GBM cells with and without stem cell qualities, and it significantly increased survival times in xenograft models. A synergistic anticancer activity in combination with TMZ and an associated biomarker for these effects furthermore suggested unique perspectives for the clinical development of niclosamide as an addition to current standards in the primary therapy of GBM.

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30 Thus, the problem underlying the present invention is solved by the provision of a combination of a first cytostatic compound according to formula I, II or III

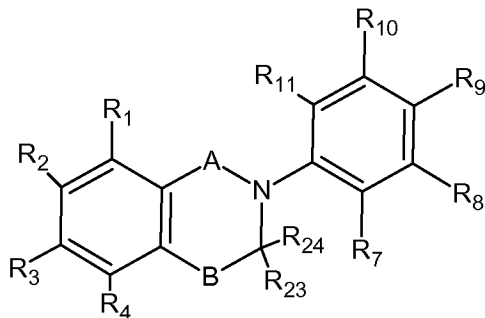
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(I)



(II)



(III)

5

wherein

- 10 A is carbonyl, methylene, hydroxymethinyl, alkoxymethinyl, aminomethinyl, oxime, hydrazone, arylhydrazone, or semicarbazone, preferably carbonyl;
- B if present is $CR_{25}R_{26}$, O, S or NR_{27} ;
- R_1 , R_3 , R_4 , R_8 , R_{10} , and R_{11} are independently hydrogen; hydroxyl; alkoxy, preferably C_1 to C_6 alkoxy; halogen, preferably fluorine, chlorine, or bromine; or C_1 to C_6 alkyl, i.e. C_1 ,
 15 C_2 , C_3 , C_4 , C_5 , or C_6 -alkyl, preferably in each case hydrogen;
- R_2 and R_7 are independently halogen, hydroxyl or hydrogen, preferably in each case halogen;
- R_5 is hydroxyl, phosphate, hydrogen; halogen, preferably fluorine, chlorine, or bromine; alkyl, preferably C_1 to C_6 alkyl, i.e. C_1 , C_2 , C_3 , C_4 , C_5 , or C_6 -alkyl; alkenyl, preferably C_2 to C_6 alkenyl; alkynyl, preferably C_2 to C_6 alkynyl; cycloalkyl, preferably C_3 to C_8

- cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl; or aryl, preferably C₆ to C₁₄ aryl, in particular phenyl; alkoxy, preferably C₁ to C₆ alkoxy; alkylthio, preferably C₁ to C₆ alkthio or amino; most preferably hydroxyl or phosphate;
- 5 R₆ if present is hydrogen or C₁ to C₆ alkyl;
- R₉ is nitro, hydrogen, hydroxyl, amino; halogen, preferably fluorine, chlorine, or bromine; alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; alkenyl, preferably C₂ to C₆ alkenyl; alkynyl, preferably C₂ to C₆ alkynyl; or aryl, preferably C₆ to C₁₄ aryl, preferably phenyl; most preferably nitro; and
- 10 R₂₁, R₂₂, R₂₃, and R₂₄, if present, are independently hydrogen; hydroxyl; halogen, preferably fluorine, chlorine, or bromine; alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; alkenyl, preferably C₂ to C₆ alkenyl; alkynyl, preferably C₂ to C₆ alkynyl; cycloalkyl, preferably C₃ to C₈ cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl; or aryl, preferably C₆ to C₁₄ aryl, in particular phenyl;
- 15 R₂₅ and R₂₆ if present are independently hydrogen, hydroxyl, halogen, preferably fluorine, chlorine, or bromine; alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; alkenyl, preferably C₂ to C₆ alkenyl; alkynyl, preferably C₂ to C₆ alkynyl; cycloalkyl, preferably C₃ to C₈ cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl; or aryl, preferably C₆ to C₁₄ aryl, in particular phenyl; and
- 20 R₂₇ if present is hydrogen or C₁ to C₆ alkyl;
- or salt thereof;
- and an alkylating compound for use in treating of a solid tumor.

A compound is a "cytostatic compound" as referred to in the present application, if it is

25 capable of inhibiting the proliferation of tumor cells. Preferably, a cytostatic compound is also cytotoxic, i.e. it actually kills the tumor cells. Preferably, the aforementioned effects of a given concentration of a cytostatic compound affect tumor cells stronger than non-tumor cells so that healthy tissue is not damaged or less damaged by said compounds as compared to tumor tissue. A preferred method for determining the strength of the cytotoxic effect of a

30 compound is the determination of the concentration which decreases the metabolic activity of a cell population to 50% of the activity of a control (ICM₅₀). This can, e.g., be done by applying resazurin to the cell culture. In healthy cells resazurin is converted to the fluorescent product resorufin. The rate of conversion can be used to measure the metabolic activity of a cell. Preferably, the cytostatic compound according to the present invention has an ICM₅₀

concentration which is not larger than 2-fold, 5-fold, 10-fold, 20-fold or 50-fold than the ICM₅₀ concentration of niclosamide or niclosamide and an alkylating agent in the same setting.

5 In a preferred embodiment of the present invention, the compound is defined by formula I, wherein

A is carbonyl;

R₁, R₃, R₄, R₈, R₁₀, and R₁₁ are independently hydrogen, hydroxyl, halogen or C₁ to C₆ alkyl, preferably in each case hydrogen;

10 R₂ and R₇ are independently halogen, hydroxyl or hydrogen, preferably in each case halogen, more preferably fluorine, chlorine or bromine, most preferably chlorine;

R₅ is hydroxyl, phosphate; hydrogen; halogen, preferably fluorine, chlorine, or bromine; alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; alkenyl, preferably C₂ to C₆ alkenyl, i.e. C₂, C₃, C₄, C₅, or C₆-alkenyl; alkynyl, preferably C₂ to C₆ alkynyl, i.e. C₂, C₃, C₄, C₅, or C₆-alkynyl; cycloalkyl, preferably C₃ to C₈ cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl; or aryl, preferably C₆ to C₁₄ aryl, in particular phenyl; alkoxy, preferably C₁ to C₆ alkoxy; alkylthio, preferably C₁ to C₆ alkthio or amino; most preferably hydroxyl or phosphate; and

15 R₉ is nitro, hydrogen, hydroxyl, amino, halogen, alkyl, alkenyl, alkynyl, or aryl, preferably nitro.

In a preferred embodiment of the present invention, the compound is defined by formula I, wherein

A is carbonyl;

25 R₁, R₃, R₄, R₈, R₁₀, and R₁₁ are independently hydrogen; hydroxyl; halogen, preferably fluorine, chlorine, or bromine; or C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; preferably in each case hydrogen;

R₂ and R₇ are independently halogen, preferably fluorine, chlorine, or bromine; hydroxyl or hydrogen; most preferably chlorine;

30 R₅ is hydroxyl, phosphate, hydrogen, halogen, preferably fluorine, chlorine, or bromine; alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; alkenyl, preferably C₂ to C₆ alkenyl, i.e. C₂, C₃, C₄, C₅, or C₆-alkenyl; alkynyl, preferably C₂ to C₆ alkynyl, i.e. C₂, C₃, C₄, C₅, or C₆-alkynyl; cycloalkyl, preferably C₃ to C₈ cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl; or aryl, preferably

C₆ to C₁₄ aryl, in particular phenyl; alkoxy, preferably C₁ to C₆ alkoxy, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkoxy; alkylthio, preferably C₁ to C₆ alkthio, i.e. i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkylthio; or amino; most preferably hydroxyl or phosphate; and

R₉ is nitro.

5

Even more preferably in the compound defined by formula I

A is carbonyl;

R₁, R₃, R₄, R₆, R₈, R₁₀ and R₁₁ are hydrogen;

R₂ and R₇ are chlorine,

10 R₅ is hydroxyl; and R₉ is nitro.

In another preferred embodiment of the present invention, the compound is defined by formula I, wherein

A is carbonyl;

15 R₁, R₃, R₄, R₆, R₈, R₁₀ and R₁₁ are hydrogen;

R₂ and R₇ are chlorine,

R₅ is phosphate; and R₉ is nitro.

In the most preferred embodiment the cytostatic compound is niclosamide.

20

It has been shown that a phosphate group significantly increases the resorption of the compound of formula I (Pan et al., 2012, Niclosamide, an old antihelminthic agent, demonstrates antitumor activity by blocking multiple signalling pathways of cancer stem cells, Vol. 31: 178-184), thus improving the bioavailability of this compound.

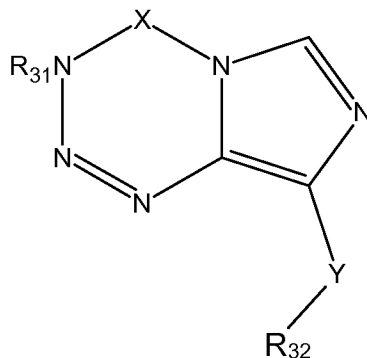
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The term "alkylating agent" relates to any pharmaceutical compound which is capable of transforming guanine to O⁶-alkylguanine, O⁴-alkylguanine or N⁷-alkylguanine, i.e. a O⁶-alkylating agent, O⁴-alkylating agent or a N⁷-alkylating agent. Suitable alkylating agents are known to the skilled person. Typically, the alkyl group added by the alkylating agent is methyl. This transformation damages the DNA and triggers the death of the cell in question. This effect affects primarily rapidly dividing cells such as cancer cells. Alkylated guanine can be detected as described by Reh et al. (1999) "O⁶-methylguanine DNA adducts associated with occupational nitrosamine exposure", Carcinogenesis, 21: 29-33. Preferably, the alkylating agent is an O⁶-alkylating agent, i.e. it exclusively or primarily alkylates the C⁶-

30

atom of guanine.

In an aspect of the invention, the alkylating compound has a structure according to formula IV



(IV)

wherein

- 10 X and Y are independently carbonyl, methylene, hydroxymethinyl, alkoxymethinyl, aminomethinyl, oxime, hydrazone, arylhydrazone or semicarbazone, preferably carbonyl, R₃₁ is alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; hydrogen; alkoxy, preferably C₁ to C₆ alkoxy, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkoxy, alkenyl, preferably C₂ to C₆ alkenyl, i.e. C₂, C₃, C₄, C₅, or C₆-alkenyl; alkynyl, preferably C₂ to C₆ alkynyl, i.e. C₂,
 15 C₃, C₄, C₅, or C₆-alkynyl; cycloalkyl, preferably C₃ to C₈ cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl; or aryl, preferably C₆ to C₁₄ aryl, in particular phenyl; preferably alkyl; and
 R₃₂ is amino, hydrogen, hydroxyl or halogen, preferably fluorine, chlorine, or bromine; or salt thereof.

- 20 In a preferred embodiment the alkylating agent is defined by formula IV X is carbonyl and Y is carbonyl, methylene, hydroxymethinyl, alkoxymethinyl, aminomethinyl, oxime, hydrazone, arylhydrazone or semicarbazone, preferably carbonyl,
 R₃₁ is alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl; hydrogen; alkoxy, preferably C₁ to C₆ alkoxy, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkoxy, alkenyl, preferably C₂
 25 to C₆ alkenyl, i.e. C₂, C₃, C₄, C₅, or C₆-alkenyl; alkynyl, preferably C₂ to C₆ alkynyl, i.e. C₂, C₃, C₄, C₅, or C₆-alkynyl; cycloalkyl, preferably C₃ to C₈ cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl; or aryl, preferably C₆ to C₁₄ aryl, in particular phenyl; preferably alkyl; and

R₃₂ is amino, hydrogen, hydroxyl or halogen, preferably fluorine, chlorine, or bromine; or salt thereof.

In an especially preferred embodiment the alkylating agent is defined by formula IV, wherein

5

X and Y are carbonyl;

R₃₁ is alkyl, preferably C₁ to C₆ alkyl, i.e. C₁, C₂, C₃, C₄, C₅, or C₆-alkyl, preferably methyl; and

R₃₂ is amino.

10

In the most preferred embodiment the alkylating agent is temozolomide.

The terms used above have the following preferred meanings:

15 Alkyl groups are, preferably, straight-chained or branched C₁ to C₁₀ alkyl groups, more preferably C₁ to C₆ alkyl groups. Alkyl groups comprising not more than 6 carbon atoms are referred to as "lower alkyl". Preferred alkyl groups comprise methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, 3-pentyl, hexyl and octyl groups.

20 Preferred cycloalkyl groups are C₃ to C₈ cycloalkyl, in particular cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl. Further preferred are the corresponding cycloalkenyl groups, in particular cyclopentenyl, cyclohexenyl, cycloheptenyl and cyclooctenyl.

Preferred alkenyl groups are C₂ to C₁₀ alkenyl, in particular ethenyl, 1- or 2-propenyl, 1-, 2- or
25 3-butenyl, 1-, 2-, 3- or 4-pentenyl, 1-, 2-, 3-, 4- or 5-hexenyl, 1-, 2-, 3-, 4-, 5- or 6-heptenyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-oktenyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- or 8-nonenyl or 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8- or 9-decenyl.

Preferred alkynyl groups are C₂ to C₁₀ alkynyl, in particular ethenyl, 1- or 2-propynyl, 1-, 2-
30 or 3-butylnyl, 1-, 2-, 3- or 4-pentylnyl, 1-, 2-, 3-, 4- or 5-hexynyl, 1-, 2-, 3-, 4-, 5- or 6-heptynyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-oktylnyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- or 8-nonylnyl or 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8- or 9-decynyl.

The terms "alkyl group", "cycloalkyl group", "alkenyl group" and "alkynyl group" also refer

to substituted alkyl, cycloalkyl, alkenyl and alkynyl groups. Preferred substituents of the said groups comprise at least one halogen, hydroxyl, carboxyl, alkoxy carbonyl, amino, nitro, cyano, C₁ to C₆ acylamino, C₁ to C₆ aminoacyl, C₁ to C₆ acyloxy, C₁ to C₆ alkoxy, aryloxy, alkylthio, C₆ to C₁₀ aryl, C₄ to C₇ cycloalkyl, C₂ to C₆ alkenyl and C₂ to C₆ alkynyl.

5

Preferred alkoxy groups comprise oxygen substituted by one of the alkyl, alkenyl or alkynyl groups recited above, preferably by methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, 3-pentyl, hexyl and octyl groups, more preferably methyl.

10 Preferred alkylthio groups comprise sulphur substituted by one of the alkyl, alkenyl or alkynyl groups recited above, preferably by methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, 3-pentyl, hexyl and octyl groups, more preferably methyl, sulfoxides and sulfones.

Preferred amino groups comprise -NH₂, -NHR₅₁, -NR₅₁R₅₂, wherein R₅₁ and R₅₂ are C₁ to C₁₀ alkyl, preferably selected from the groups methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, 3-pentyl, hexyl and octyl groups, most preferably methyl, or cycloalkyl groups or R₅₁ and R₅₂ are combined with the N to form a ring structure, preferably a 5 to 7 membered ring structure, such as piperidine or R₅₁ and R₅₂ are combined with the N and another heteroatom to form a saturated, substituted, or partially saturated 5-7-membered heterocyclic group. Preferred heteroatoms include O, N and S.

20

Preferred aryl groups comprise C₆ to C₁₄ aryl, more particularly C₆ to C₁₀ aryl. More preferably, the aryl group is phenyl, naphthyl, phenanthrenyl, anthracenyl, indenyl, azulenyl, bphenyl, biphenylenyl or fluorenyl. The term "aryl group" also refers to substituted aryl groups as defined below.

25

Preferred substituents of the aryl, groups comprise at least one acyl, alkylenedioxy (-OCH₂O-), halogen, C₆ to C₁₀ aryl, C₄ to C₇ cycloalkyl, C₁ to C₆ alkyl, C₂ to C₆ alkenyl, C₂ to C₆ alkynyl, C₁ to C₆ hydroxyalkyl, nitro, amino or C₁ to C₆ alkoxy group.

30

Preferred halogens comprise fluorine, iodine, chlorine and bromine.

In another preferred embodiment, the alkylating agent is selected from the group consisting of nitrogen mustards, nitrosoureas, alkyl sulfonates, platinum-based chemotherapeutic drugs and

non-classical alkylating agents.

Preferred nitrogen mustards are cyclophosphamide, mechlorethamine, uramustine, melphalan, chlorambucil, trofosfamide and ifosfamide.

5

Preferred nitrosoureas are carmustine, lomustine and streptozocin.

A preferred alkyl sulfonate is busulfan.

10 Preferred platinum-based chemotherapeutic drugs are cisplatin, carboplatin, nedaplatin, oxaliplatin, satraplatin and triplatin tetranitrate.

Preferred non-classical alkylating agents are procarbazine, altetramine, dacarbazine, thiotepa and mitozolomide. Particularly preferred are dacarbazine and mitozolomide.

15

Most preferably, the alkylating agent is a nitrosourea, in particular carmustine.

The term „solid tumor“ refers to any coherent group of neoplastic cells. A „solid tumor“ according to the present application may be benign, pre-malign or malign. Preferably, it is
20 pre-malign or malign and, most preferably, malign.

20

Preferred pre-malign tumors are selected from the group consisting of actinic keratosis, cutaneous horn, actinic cheilitis, tar keratosis, arsenic keratosis, x-ray keratosis, Bowen's disease, bowenoid papulosis, lentigo maligna, lichen sclerosus, and lichen rubber mucosae;
25 precancerosis of the digestive tract, in particular erythroplakia, leukoplakia, Barrett's esophagus, Plummer-Vinson syndrome, crural ulcer, gastropathia hypertrophica gigantea, borderline carcinoma, neoplastic intestinal polyp, rectal polyp, porcelain gallbladder; gynaecological precancerosis, in particular carcinoma ductale in situ (CDIS), cervical intraepithelial neoplasia (CIN), leukoplakia, endometrial hyperplasia (grade III), vulvar
30 dystrophy, vulvar intraepithelial neoplasia (VIN), hydatidiform mole; urologic precancerosis, in particular bladder papillomatosis, Queyrat's erythroplasia, testicular intraepithelial neoplasia (TIN), leukoplakia and carcinoma in situ (CIS).

Preferred malign tumors are selected from the group consisting of glioblastoma, diffuse large

B-cell lymphoma (DLBCL), T-cell lymphomas, e.g., cutaneous T-cell lymphoma (CTCL), noncutaneous peripheral T-cell lymphoma, lymphoma associated with human T-cell lymphotropic virus (HTLV), adult T-cell lymphoma (ATLL), Hodgkin's lymphoma, non-Hodgkin's lymphoma (including primary central nervous system lymphoma), multiple myeloma, multiple myeloma, mesothelioma, childhood solid tumors, bone cancer and soft-tissue sarcomas, common solid tumors of adults such as head and neck cancers (e.g., oral, laryngeal and esophageal), genitourinary cancers (e.g., prostate, bladder, renal (in particular malignant renal cell carcinoma (RCC)), uterine, ovarian, testicular), colorectal carcinoma, lung cancer (e.g., small cell carcinoma and non-small cell lung carcinoma, including squamous cell carcinoma and adenocarcinoma), breast cancer, pancreatic cancer, melanoma and other skin cancers, basal cell carcinoma, metastatic skin carcinoma, squamous cell carcinoma of both ulcerating and papillary type, stomach cancer, brain cancer, hepatocellular carcinoma, adrenal cancer, kidney cancer, thyroid cancer, medullary carcinoma, osteosarcoma, soft-tissue sarcoma, Ewing's sarcoma, veticulum cell sarcoma, and Kaposi's sarcoma, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, seminoma, embryonal carcinoma, Wilms' tumor, small cell lung carcinoma, epithelial carcinoma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, hemangioma, heavy chain disease and metastases.

25

In a preferred embodiment of the present invention, the solid tumor is selected from the group consisting of glioblastoma, Hodgkin's lymphoma, colorectal cancer, melanoma hepatocellular carcinoma, breast cancer and multiple myeloma. Most preferably, the solid tumor is glioblastoma.

30

The term "glioblastoma" or "glioblastoma multiforme" refers to a primary brain tumor involving glial cells. Glioblastoma is, preferably, diagnosed based on the histological presence of proliferative glial tumor cells, vascular proliferation and preferentially necrotic tissue areas (for further classification: Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Burger,

P.C., Jouvett, A., Scheithauer, B.W., and Kleihues, P. 2007. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 114:97-109.)

In a more preferred embodiment of the present invent, the glioblastoma is selected from the group consisting of primary glioblastoma, secondary glioblastoma, de novo glioblastoma, recurrent glioblastoma, glioblastoma with increased methylation of the promoter of the gene O⁶-Methylguanin-Methyltransferase (MGMT), glioblastoma without increased methylation of the promoter of MGMT, glioblastoma with mutated p53, glioblastoma without mutated p53, glioblastoma with alterations of the gene encoding kappa light polypeptide gene enhancer in B-cells inhibitor (NFKBIA), glioblastoma without alterations of the gene encoding NFKBIA, glioblastoma with alterations of the gene encoding epidermal growth factor receptor (EGFR), glioblastoma without alterations of the gene encoding EGFR, glioblastoma with alterations of the gene encoding platelet-derived growth factor receptor (PDGFRA), glioblastoma without alterations of the gene encoding PDGFRA, glioblastoma with alterations of the gene encoding isocitrate dehydrogenase 1 (IDHI), glioblastoma without alterations of the gene encoding IDHI, glioblastoma with alterations of the gene encoding neurofibromatosis type 1 (NF1) and glioblastoma without alterations of the gene encoding NF1.

The term “alterations” refers to mutations, deletions and the presence of additional copies of the gene in question. Mutations and deletions may be homo- or heterozygous. Moreover, the term “glioblastoma” refers to primary as well as recurrent disease.

Particularly preferred is the treatment of a solid tumor, in particular a solid tumor selected from the group consisting of glioblastoma, Hodkin’s lymphoma, colorectal cancer, melanoma hepatocellular carcinoma, breast cancer and multiple myeloma, wherein said tumor is characterized by underexpression of NFKBIA, with a compound as defined by formula I, II or III in combination with an alkylating agent. Preferably, said underexpression of NFKBIA is caused by a heterozygous deletion of the NFKBIA locus (NFKBIA^{+/-}) at 14q13.

The compound according to formula I, II or III and the alkylating agent are, preferably, administered simultaneously or subsequently.

The term “simultaneously” refers to the administration of dosages of both compounds which is not separated by more than 5 minutes, more than 10 minutes or more than 20 minutes.

More preferably, both compounds are components comprised by a single pharmaceutical composition which is administered to the patient.

5 The term “subsequent administration” refers to an administration regimen where the interval between the administration of both compounds is chosen to enable or, more preferably, maximize the synergistic effect of the combination. Preferably, the interval between the administration of the compound according to formula I, II or III and the alkylating agent is chosen to achieve simultaneous peak plasma levels of both substances. Also preferably, the interval between the administration of the compound according to formula I, II or III and the
10 alkylating agent does not exceed 1 day, 3 days, 6 days or 9 days.

The administered amounts of compounds are, preferably chosen so that defined ratios of the active compounds at the site of the tumor are achieved. It is known to the person skilled in the art that the effective concentration of a pharmaceutical compound at the site of a tumor
15 depends on the route of administration, the distribution of the compound in different compartments (e.g. blood and tissue), the enzymatic activation of a prodrug and/or the enzymatic inactivation of the active compound, and the rate of excretion of the compound from the body. Thus, the decision about a suitable dosage depends on the aforementioned parameters. However, the person skilled in the art is well aware of this issue and
20 pharmacokinetic analysis helps in determining a dosage of a compound which leads to the desired concentration of the active compound at the site of the tumor.

Preferred routes of administration are oral, intravenous, intrathecal intraparenchymal administration.

25 Preferably, at the site of the tumor the molar ratio of niclosamide (expressed as mol % of the added molar concentrations of niclosamide and temozolomide) is 10 mol%, 20 mol%, 30 mol %, 40 mol%, 50 mol%, 60 mol%, 70 mol%, 80 mol% or 90 mol%. More preferably, the molar ratio of both compounds depends on the expression status of NFKBIA.

30 If the tumor in question is characterized by a decreased expression of NFKBIA, the molar ratio of niclosamide is, preferably, equal to larger than 40% of the added molar concentrations of niclosamide and temozolomide. More preferably, the concentration is larger than 50 mol%, larger than 60 mol%, larger than 70 mol%, larger than 80 mol% or larger than 90 mol%, most

preferably it is 50 mol%.

If the tumor in question is not characterized by a decreased expression of NFKBIA, the molar ratio of niclosamide is, preferably, smaller than 40% of the added molar concentrations of niclosamide and temozolomide. More preferably, the concentration is smaller than 40 mol%,
5 smaller than 30 mol%, smaller than 20 mol% or smaller than 10 mol%, most preferably is 10%.

The term “treating” refers to the administration of the compounds of the present invention to a
10 patient with the aim of restoring or maintaining the patient’s health with respect to a solid tumor.

In a preferred embodiment of the present invention, the treatment is curative. Preferably, curative treatment aims at the complete eradication of all tumor cells from the patient’s body.
15 It is to be understood that curative treatment may not be successful in all patients receiving the treatment. Almost no medical therapy in general, and almost no cancer therapy in particular, works in each and every patient. However, curative treatment means that a significantly larger portion of patients can be cured as compared to groups patients receiving no treatment or only a placebo. Statistical methods for determining whether the rate of success
20 is higher in one group of patients as compared to another one are well known to the person skilled in the art.

In another preferred embodiment of the present invention the treatment is palliative. Palliative treatment does not aim at the complete eradication of all tumor cells from the patient’s body
25 although such an outcome is not excluded by the term “palliative treatment”. Preferably, palliative treatment aims at the reduction of tumor associated symptoms, such as pain or neurological deficiencies, in the patient. Also preferably, palliative treatment aims at decreasing tumor bulk and symptoms associated therewith, increasing the time until tumor progression or increasing the time of survival. Furthermore, it is preferred that “palliative
30 treatment” keeps the tumor stable, i.e. prevents the tumor mass from increasing and the formation of new metastases.

Preferred subtypes are adjuvant or neoadjuvant treatment regimens. Both, adjuvant and neoadjuvant treatment are characterized by the administration of the compounds of the

present invention in combination with a surgical removal of one or more solid tumors.

The term “neoadjuvant treatment”, preferably, refers to the administration of the compounds of the present invention prior to surgery. Such an administration regimen may be used to
5 reduce the size of the tumor before surgery in order to increase the chance of a complete surgical removal or in order to decrease the amount of tissue that has to be resected during surgery so that surgery is less straining for the patient.

10 Preferably, the term “adjuvant treatment” refers to the administration of the compounds of the present invention after surgery. Adjuvant treatment may also be accompanied by radiotherapy. In many cases, surgery does not result in the complete resection of the tumor or of all tumors from the patient’s body. Distant metastases may be already present but too small to be detected by imaging methods. Similarly, tumor cells may already have spread locally beyond the apparent margins of the tumor. In both cases, adjuvant therapy with the compounds of the
15 present invention offers the chance to kill those tumor cells which are not removed by surgery and – where applicable – radiotherapy, thus improving the chance of a complete cure.

Both, adjuvant as well as neoadjuvant treatment may be administered as curative as well as
20 palliative treatment.

In some cases, a tumor is not amenable to surgery either because the patient is so weak that surgery has to be considered an unacceptable risk or because the tumor is not accessible to surgery without damaging vital anatomical structures of the patient. Both situations frequently occur in patients with metastasized tumors. In such cases the advanced stage of the cancer
25 disease weakens the patient considerably and, at the same time, the many tumors present in the body would require extensive surgery. In these cases it is preferred that the compound according to formula I, II or III alone or in combination with the alkylating agent is administered as systemic treatment without surgery. The person skilled in the art knows that medical treatment of a tumor typically relies on the combination of 2, 3 or even more
30 pharmaceutical compounds. Therefore, the compound according to formula I, II or III alone or the combination of the compound according to formula I, II or III and the alkylating agent may be complemented with further cytostatic and/or cytotoxic compounds.

In yet another preferred embodiment of the present invention, the compounds of the present

invention the treatment aims at preventing recurrence of an apparently successfully treated tumor. Frequently, treatment of a tumor by means of surgery, radiation therapy, chemotherapy or a combination of any of the aforementioned therapies induces a remission of the tumor. Remission of a tumor is a state, where no traces of a tumor are detectable clinically, e.g. by
5 imaging methods, visual inspection or laboratory parameters. However, in many cases tumors recur at the same location as the original tumor(s) or at distant body parts within a few years after apparently successful treatment. Therefore, remission of a tumor does not equal a successful cure. The compounds of the present invention may be administered in order to prevent recurrence of an apparently successfully treated tumor independent of the primary
10 therapy chosen.

The patient is, preferably, a vertebrate, more preferably, a mammal. Even more preferably, the patient is a cat, dog, monkey, sheep, goat, pig, mouse, rat or human. Most preferably, the patient is a human.

15 In an embodiment of the present invention, the compounds outlined above are present as pharmaceutically acceptable salts. The term "pharmaceutically acceptable salt" refers to a salt of the compound of the present invention. Suitable pharmaceutically acceptable salts of the compound of the present invention include acid addition salts which may, for example, be
20 formed by mixing a solution of the compounds of the present invention with a solution of a pharmaceutically acceptable acid such as hydrochloric acid, sulfuric acid, fumaric acid, maleic acid, succinic acid, acetic acid, benzoic acid, citric acid, tartaric acid, carbonic acid or phosphoric acid. Furthermore, where the compound of the invention carries an acidic moiety, suitable pharmaceutically acceptable salts thereof may include alkali metal salts (e.g., sodium
25 or potassium salts); alkaline earth metal salts (e.g., calcium or magnesium salts); and salts formed with suitable organic ligands (e.g., ammonium, quaternary ammonium and amine cations formed using counteranions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, alkyl sulfonate and aryl sulfonate). Illustrative examples of pharmaceutically acceptable salts include but are not limited to: acetate, adipate, alginate,
30 ascorbate, aspartate, benzenesulfonate, benzoate, bicarbonate, bisulfate, bitartrate, borate, bromide, butyrate, calcium edetate, camphorate, camphorsulfonate, camsylate, carbonate, chloride, citrate, clavulanate, cyclopentanepropionate, digluconate, dihydrochloride, dodecylsulfate, edetate, edisylate, estolate, esylate, ethanesulfonate, formate, fumarate, gluceptate, glucoheptonate, gluconate, glutamate, glycerophosphate, glycolylarsanilate,

hemisulfate, heptanoate, hexanoate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, hydroxynaphthoate, iodide, isothionate, lactate, lactobionate, laurate, lauryl sulfate, malate, maleate, malonate, mandelate, mesylate, methanesulfonate, methylsulfate, mucate, 2-naphthalenesulfonate, napsylate, 5 nicotinate, nitrate, N-methylglucamine ammonium salt, oleate, oxalate, pamoate (embonate), palmitate, pantothenate, pectinate, persulfate, 3-phenylpropionate, phosphate/diphosphate, picrate, pivalate, polygalacturonate, propionate, salicylate, stearate, sulfate, subacetate, succinate, tannate, tartrate, teoate, tosylate, triethiodide, undecanoate, valerate, and the like (see, for example, Berge, S. M., et al, "Pharmaceutical Salts", *Journal of Pharmaceutical* 10 *Science*, 1977, 66, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The neutral forms of the compounds may be regenerated by contacting the salt with a base or 15 acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

20 In addition to salt forms, the present invention provides compounds which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide a compound of general formula (I) – (III). A prodrug is a pharmacologically active or inactive compound that is modified chemically through *in vivo* physiological action, such as hydrolysis, metabolism and the like, 25 into a compound of this invention following administration of the prodrug to a patient. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme. The suitability and techniques involved in making and 30 using prodrugs are well known by those skilled in the art. For a general discussion of prodrugs involving esters, see Svensson and Tunek, *Drug Metabolism Reviews* 16.5 (1988), and Bundgaard, *Design of Prodrugs*, Elsevier (1985). Examples of a masked acidic anion include a variety of esters, such as alkyl (for example, methyl, ethyl), cycloalkyl (for example, cyclohexyl), aralkyl (for example, benzyl, p-methoxybenzyl), and alkylcarbonyloxyalkyl (for

example, pivaloyloxymethyl). Amines have been masked as arylcarbonyloxymethyl substituted derivatives which are cleaved by esterases *in vivo* releasing the free drug and formaldehyde (Bungaard J. Med. Chem. 2503 (1989)). Also, drugs containing an acidic NH group, such as imidazole, imide, indole and the like, have been masked with N-acyloxymethyl groups (Bundgaard Design of Prodrugs, Elsevier (1985)). Hydroxy groups have been masked as esters and ethers. EP 0 039 051 (Sloan and Little, Apr. 11, 1981) discloses Mannich-base hydroxamic acid prodrugs, their preparation and use.

Compounds of the present invention and also the starting materials for their preparation according to the invention can be synthesized as shown herein, and, alternatively, by methods and standard procedures known to those skilled in the art, i. e. as described in the literature (for example in the standard works, such as Houben-Weyl, Methoden der organischen Chemie [Methods of Organic Chemistry], Georg-Thieme-Verlag, Stuttgart), under reaction conditions which are known to those skilled in the art and suitable for the said reactions.

If desired, the starting materials can also be formed *in situ* by not isolating them from the reaction mixture, but instead immediately converting them further into the compounds of the present invention. On the other hand, it is possible to carry out the reaction stepwise. It should be noted that the general procedures are shown as it relates to preparation of compounds having unspecified stereochemistry. However, such procedures are generally applicable to those compounds of a specific stereochemistry, e.g., where the stereochemistry at a stereogenic center is (S) or (R). In addition, the compounds having one stereochemistry (e.g., (R)) can often be utilized to produce those having opposite stereochemistry (i.e., (S)) using well-known methods, for example, by inversion.

Certain compounds of the present invention can exist in unsolvated forms as well as in solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are intended to be encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds. The racemates, enantiomers, diastereomers, geometric isomers and

individual isomers are all intended to be encompassed within the scope of the present invention. Accordingly, the compounds of this invention include mixtures of stereoisomers, especially mixtures of enantiomers, as well as purified stereoisomers, especially purified enantiomers, or stereoisomerically enriched mixtures, especially enantiomerically enriched mixtures. Also included within the scope of the invention are the individual isomers of the compounds represented by formulas (I) to (IV) below as well as any wholly or partially equilibrated mixtures thereof. The present invention also covers the individual isomers of the compounds represented by the formulas below as mixtures with isomers thereof in which one or more chiral centers are inverted. Also, it is understood that all tautomers and mixtures of tautomers of the compounds of formulas (I) to (IV) are included within the scope of the compounds of formulas (I) to (IV) and preferably the formulas and subformulas corresponding thereto.

Racemates obtained can be resolved into the isomers mechanically or chemically by methods known per se. Diastereomers are preferably formed from the racemic mixture by reaction with an optically active resolving agent.

Examples of suitable resolving agents are optically active acids, such as the D and L forms of tartaric acid, diacetyltartaric acid, dibenzoyltartaric acid, mandelic acid, malic acid, lactic acid or the various optically active camphorsulfonic acids, such as -camphorsulfonic acid. Also advantageous is enantiomer resolution with the aid of a column filled with an optically active resolving agent (for example dinitrobenzoylphenylglycine); an example of a suitable eluent is a hexane/isopropanol/acetonitrile mixture.

The diastereomer resolution can also be carried out by standard purification processes, such as, for example, chromatography or fractional crystallization.

It is also possible to obtain optically active compounds of formulas (I) to (IV) by the methods described above by using starting materials which are already optically active.

In a further aspect the present invention relates to a pharmaceutical composition comprising a compound according to formula I, II or III, wherein A and R₁ to R₁₁ have the meaning and preferred meanings set out above, and a pharmaceutically acceptable excipient for use in treating of a solid tumor. In a preferred embodiment, the pharmaceutical composition

additionally comprises an alkylating agent, preferably as defined by formula IV, wherein X, Y, R₃₁ and R₃₂ have the meaning and preferred meanings set out above.

5 A "pharmaceutical composition" as referred to in the present application comprises at least one compound of the present invention and at least one pharmaceutically acceptable carrier.

For preparing pharmaceutical compositions from the compounds of the present invention, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A
10 solid carrier can be one or more substances, which may also act as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material.

In powders, the carrier is a finely divided solid, which is in a mixture with the finely divided active component. In tablets, the active component is mixed with the carrier having the
15 necessary binding properties in suitable proportions and compacted in the shape and size desired.

The powders and tablets preferably contain from 5% to 80%, more preferably from 20% to 70% of the active compound or active compounds. Suitable carriers are magnesium
20 carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like.

The term "preparation" is intended to include the formulation of the active compound with
25 encapsulating material as a carrier providing a capsule in which the active component with or without other carriers, is surrounded by a carrier, which is thus in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid dosage forms suitable for oral administration.

30 For preparing suppositories, a low melting wax, such as a mixture of fatty acid glycerides or cocoa butter, is first melted and the active component is dispersed homogeneously therein, as by stirring. The molten homogeneous mixture is then poured into convenient sized molds, allowed to cool, and thereby to solidify.

Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water/propylene glycol solutions. Liquid forms are particularly preferred for topical applications to the eye. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

5

Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizers, and thickening agents as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

10

Also included are solid form preparations, which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

15

The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

20

In a preferred embodiment of the present invention, the pharmaceutical composition additionally comprises at least one further cytostatic or cytotoxic compound. Said additional compound is selected from the group consisting of Temozolimide, Daunorubicine, Gambogic acid amide, Gambogic acid, Thimerosal, Mitoxanthrone hydrochloride, Phenylmercuric acetate, Dactinomycin, Pristimerin, Epirubicin hydrochloride, Vincristine sulfate, Emetine, Paclitaxel, 10-Hydroxycamptothecin, Doxorubicine, Colchicine, Camptothecin, Teniposide, Vinblastine sulfate, Mitomycin C, Floxuridine, Ouabain, Ancitabine hydrochloride, Quinacrine hydrochloride, Niclosamide, Amsacrine, Thioguanine, Rotenone, Aklavine hydrochloride, Cytarabine, Methotrexate, and Picropodophyllotoxin.

30

Further preferred additional cytostatic or cytotoxic compounds include anti-estrogens such as faslodex, tamoxifen or raloxifen; any inhibitors of topoisomerase I or II, such as camptothecin (topo I) or etoposide (topo II); any compound that acts through inhibiting aromatase activity, such as anastrozole or letrozole; any preparation that interferes with HER2 signalling such as hereceptin; any compound that interchelates DNA, such as doxorubicin. Particularly preferred 5 cytostatic or cytotoxic drugs, which can be combined with the compounds of the present invention are alkylating substances, anti-metabolites, antibiotics, epothilones, nuclear receptor agonists and antagonists, anti-androgens, anti-estrogens, platinum compounds, hormones and antihormones, interferons and inhibitors of cell cycle-dependent protein kinases (CDKs), 10 inhibitors of cyclooxygenases and/or lipoxygenases, biogenic fatty acids and fatty acid derivatives, including prostanoids and leukotrienes, inhibitors of protein kinases, inhibitors of protein phosphatases, inhibitors of lipid kinases, platinum coordination complexes, ethyleneimines, methylmelamines, trazines, vinca alkaloids, pyrimidine analogs, purine analogs, alkylsulfonates, folic acid analogs, anthracendiones, substituted urea, methylhydrazin 15 derivatives, in particular acediasulfone, aclarubicine, ambazone, aminoglutethimide, L-asparaginase, azathioprine, bleomycin, busulfan, calcium folinate, carboplatin, carpecitabine, carmustine, celecoxib, chlorambucil, cis-platin, cladribine, cyclophosphamide, cytarabine, dacarbazine, dactinomycin dapsone, daunorubicin, dibrompropamide, diethylstilbestrol, docetaxel, doxorubicin, enediyne, epirubicin, epothilone B, epothilone D, estramucine 20 phosphate, estrogen, ethinylestradiol, etoposide, flavopiridol, floxuridine, fludarabine, fluorouracil, fluoxymesterone, flutamide fosfestrol, furazolidone, gemcitabine, gonadotropin releasing hormone analog, hexamethylmelamine, hydroxycarbamide, hydroxymethylnitrofurantoin, hydroxyprogesteronecaproat, hydroxyurea, idarubicin, idoxuridine, ifosfamide, interferon γ , irinotecan, leuprolide, lomustine, lurtotecan, mafenide 25 sulfate olamide, mechlorethamine, medroxyprogesterone acetate, megastrolacetate, melphalan, mepacrine, mercaptopurine, methotrexate, metronidazole, mitomycin C, mitopodozide, mitotane, mitoxantrone, mithramycin, nalidixic acid, nifuratel, nifuroxazide, nifuralazine, nifurtimox, nimustine, ninorazole, nitrofurantoin, nitrogen mustards, oleomucin, oxolinic acid, pentamidine, pentostatin, phenazopyridine, phthalylsulfathiazole, pipobroman, 30 prednimustine, prednisone, preussin, procarbazine, pyrimethamine, raltitrexed, rapamycin, rofecoxib, rosiglitazone, salazosulfapyridine, scriflavinium chloride, semustine streptozocine, sulfacarbamide, sulfacetamide, sulfachlopyridazine, sulfadiazine, sulfadicramide, sulfadimethoxine, sulfaethidole, sulfafurazole, sulfaguanidine, sulfaguanole, sulfamethizole, sulfamethoxazole, co-trimoxazole, sulfamethoxydiazine, sulfamethoxypridazine,

sulfamoxole, sulfanilamide, sulfaperin, sulfaphenazole, sulfathiazole, sulfisomidine, staurosporin, tamoxifen, taxol, teniposide, tertiposide, testolactone, testosteronepropionate, thioguanine, thiotepa, tinidazole, topotecan, triaziquone, treosulfan, trimethoprim, trofosfamide, UCN-01, vinblastine, vincristine, vindesine, vinblastine, vinorelbine, and
5 zorubicin, or their respective derivatives or analogs thereof.

In a particularly preferred embodiment of the present invention the additional cytostatic or cytotoxic compound is temozolomide.

10 Salts / esters

The compounds within the compositions or compounds usable according to the present invention can be present as salts or esters, in particular pharmaceutically acceptable salts or esters. Pharmaceutically acceptable salts of the compounds of the invention include suitable acid addition or base salts thereof. A review of suitable pharmaceutical salts may be found in
15 Berge et al, J Pharm Sci, 66, 1-19 (1977). Salts are formed, for example with strong inorganic acids such as mineral acids, e.g. sulphuric acid, phosphoric acid or hydrohalic acids; with strong organic carboxylic acids, such as alkanecarboxylic acids of 1 to 4 carbon atoms which are unsubstituted or substituted (e.g., by halogen), such as acetic acid; with saturated or unsaturated dicarboxylic acids, for example oxalic, malonic, succinic, maleic, fumaric,
20 phthalic or tetraphthalic; with hydroxycarboxylic acids, for example ascorbic, glycolic, lactic, malic, tartaric or citric acid; with aminoacids, for example aspartic or glutamic acid; with benzoic acid; or with organic sulfonic acids, such as (C1-C4)-alkyl- or aryl-sulfonic acids which are unsubstituted or substituted (for example, by a halogen) such as methane- or p-toluene sulfonic acid.

25

Esters are formed either using organic acids or alcohols/hydroxides, depending on the functional group being esterified. Organic acids include carboxylic acids, such as alkanecarboxylic acids of 1 to 12 carbon atoms which are unsubstituted or substituted (e.g., by halogen), such as acetic acid; with saturated or unsaturated dicarboxylic acid, for example
30 oxalic, malonic, succinic, maleic, fumaric, phthalic or tetraphthalic; with hydroxycarboxylic acids, for example ascorbic, glycolic, lactic, malic, tartaric or citric acid; with aminoacids, for example aspartic or glutamic acid; with benzoic acid; or with organic sulfonic acids, such as (C1-C4)-alkyl- or aryl-sulfonic acids which are unsubstituted or substituted (for example, by a halogen) such as methane- or p-toluene sulfonic acid. Suitable hydroxides include inorganic

hydroxides, such as sodium hydroxide, potassium hydroxide, calcium hydroxide, aluminium hydroxide. Alcohols include alkanealcohols of 1-12 carbon atoms which may be unsubstituted or substituted, e.g. by a halogen).

5 Isotopes

The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. An isotopic variation of an agent of the present invention or a pharmaceutically acceptable salt thereof is defined as one in which at least one atom is replaced by an atom having the same atomic number but an atomic mass different from the atomic mass usually found in nature. Examples of isotopes that can be incorporated into the agent and pharmaceutically acceptable salts thereof include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorus, sulphur, fluorine and chlorine such as ^2H , ^3H , ^{13}C , ^{14}C , ^{15}N , ^{17}O , ^{18}O , ^{31}P , ^{32}P , ^{35}S , ^{18}F and ^{36}Cl , respectively. Certain isotopic variations of the agent and pharmaceutically acceptable salts thereof, for example, those in which a radioactive isotope such as ^3H or ^{14}C is incorporated, are useful in drug and/or substrate tissue distribution studies. Tritiated, i.e., ^3H , and carbon-14, i.e., ^{14}C , isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with isotopes such as deuterium, i.e., ^3H , may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased in vivo half-life or reduced dosage requirements and hence may be preferred in some circumstances. Isotopic variations of the agent of the present invention and pharmaceutically acceptable salts thereof of this invention can generally be prepared by conventional procedures using appropriate isotopic variations of suitable reagents.

25 All isotopic variations of the compounds and compositions of the present invention, whether radioactive or not, are intended to be encompassed within the scope of the present invention.

Solvates

The present invention also includes solvate forms of the compounds within the compositions or compounds according to any of general formulas (I) through (III) usable according to the present invention. The terms used in the claims encompass these forms.

Polymorphs

The invention furthermore relates to compounds within the compositions of the present

invention or compounds according to formula (I) usable according to the present invention in their various crystalline forms, polymorphic forms and (an)hydrous forms. It is well established within the pharmaceutical industry that chemical compounds may be isolated in any of such forms by slightly varying the method of purification and or isolation from the solvents used in the synthetic preparation of such compounds.

Administration

A compound according to the invention can be administered by various well known routes, including oral, rectal, intragastrical, intracranial and parenteral administration, e.g. intravenous, intramuscular, intranasal, intradermal, subcutaneous, and similar administration routes. Parenteral administration and particular intravenous administration, preferably by depot injection, is preferred. Depending on the route of administration different pharmaceutical formulations are required and some of those may require that protective coatings are applied to the drug formulation to prevent degradation of a compound of the invention in, for example, the digestive tract.

Thus, preferably, a compound of the invention is formulated as a syrup, an infusion or injection solution, a tablet, a capsule, a capslet, lozenge, a liposome, a suppository, a plaster, a band-aid, a retard capsule, a powder, or a slow release formulation. Preferably the diluent is water, a buffer, a buffered salt solution or a salt solution and the carrier preferably is selected from the group consisting of cocoa butter and vitebesole.

Particular preferred pharmaceutical forms for the administration of a compound of the invention are forms suitable for injectable use and include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the final solution or dispersion form must be sterile and fluid. Typically, such a solution or dispersion will include a solvent or dispersion medium, containing, for example, water-buffered aqueous solutions, e.g. biocompatible buffers, ethanol, polyol, such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants or vegetable oils. A compound of the invention can also be formulated into liposomes, in particular for parenteral administration. Liposomes provide the advantage of increased half life in the circulation, if compared to the free drug and a prolonged more even release of the enclosed drug.

5 Sterilization of infusion or injection solutions can be accomplished by any number of art recognized techniques including but not limited to addition of preservatives like anti-bacterial or anti-fungal agents, e.g. parabene, chlorobutanol, phenol, sorbic acid or thimersal. Further, isotonic agents, such as sugars or salts, in particular sodium chloride may be incorporated in infusion or injection solutions.

10 Production of sterile injectable solutions containing one or several of the compounds of the invention is accomplished by incorporating the respective compound in the required amount in the appropriate solvent with various ingredients enumerated above as required followed by sterilization. To obtain a sterile powder the above solutions are vacuum-dried or freeze-dried as necessary. Preferred diluents of the present invention are water, physiological acceptable buffers, physiological acceptable buffer salt solutions or salt solutions. Preferred carriers are cocoa butter and vitebesole. Besides the preferred excipients mentioned already above, also the following excipients can be chosen, without limitation, to be used with the various pharmaceutical forms of a compound of the invention:

- 15 a) binders such as lactose, mannitol, crystalline sorbitol, dibasic phosphates, calcium phosphates, sugars, microcrystalline cellulose, carboxymethyl cellulose, hydroxyethyl cellulose, polyvinyl pyrrolidone and the like;
- 20 b) lubricants such as magnesium stearate, talc, calcium stearate, zinc stearate, stearic acid, hydrogenated vegetable oil, leucine, glycerids and sodium stearyl fumarates,
- c) disintegrants such as starches, croscarmellose, sodium methyl cellulose, agar, bentonite, alginic acid, carboxymethyl cellulose, polyvinyl pyrrolidone and the like.

25 Other suitable excipients can be found in the Handbook of Pharmaceutical Excipients, published by the American Pharmaceutical Association, which is herein incorporated by reference.

30 It is to be understood that depending on the severity of the disorder and the particular type which is treatable with one of the compounds of the invention, as well as on the respective patient to be treated, e.g. the general health status of the patient, etc., different doses of the respective compound are required to elicit a therapeutic or prophylactic effect. The determination of the appropriate dose lies within the discretion of the attending physician.

As is known in the art, the pharmaceutically effective amount of a given composition will also

depend on the administration route. In general the required amount will be higher, if the administration is through the gastrointestinal tract; e.g. by suppository, rectal, or by an intragastric probe, and lower if the route of administration is parenteral, e.g. intravenous.

5 Within the meaning of this invention, a combination of substituents or variables is permissible only if such a combination results in a stable or chemically feasible compound. A stable compound or chemically feasible compound is one that is not substantially altered when kept at a temperature of 40°C or less, in the absence of moisture or other chemically reactive conditions, for at least a week. This invention also envisions the quaternization of any basic
10 nitrogen-containing groups of the compounds disclosed herein. Water or oil-soluble or dispersible products may be obtained by such quaternization.

Preferably, the compounds are formulated for oral, intravenous, intrathecal intraparenchymal administration. As both the compounds defined by formulas I, II and III and temozolomide
15 can be administered orally, it is especially preferred that both compounds are formulated for oral administration. In this case, appropriate amounts of both compounds can easily be combined for simultaneous administration so that the most effective concentrations are achieved simultaneously at the site of the tumor.

20 In the most preferred embodiment of the present invention, the compounds of formulas (I) to (IV) are formulated for resorption into the central nervous system.

In another aspect, the present invention relates to a compound according to formula I, II or III, wherein R₁ to R₁₁ and R₂₁ to R₂₇ have the meaning as defined above for use in treating of a
25 solid tumor characterized by a decreased expression level of NFKBIA.

Unless indicated otherwise, all definitions given above also apply to this aspect of the invention.

30 The NF-κB pathway is thought to play an important role in tumorigenesis and in the resistance of tumor cell to chemotherapeutic agents. As set forth above, NFKBIA binds to nuclear factor kappa B in the cytosol, thus inhibiting the translocation of NF-κB into the nucleus. In the study underlying the present invention it has been surprisingly found that niclosamide does not increase the expression of NFKBIA in tumor cells with two intact copies

of the gene while it significantly increases the expression of NFKBIA in cells with a heterozygous deletion of the gene encoding NFKBIA.

Thus, niclosamide is particularly suited for the treatment of solid tumors characterized by
5 decreased expression of NFKBIA, in particular by decreased expression caused by a heterozygous deletion of the gene encoding NFKBIA. The tumor may be any tumor recited above provided that it displays a decreased expression of NFKBIA. Preferably, the tumor is selected from the group consisting of glioblastoma, Hodkin's lymphoma, colorectal cancer, melanoma hepatocellular carcinoma, breast cancer and multiple myeloma. Most preferably,
10 the solid tumor is glioblastoma.

In a preferred embodiment of this aspect of the invention, the compound according to formula (I), (II) or (III) is combined with any cytostatic or cytotoxic compound recited above in the present application. Said combination is not limited to alkylating agents.

15

A preferred embodiment of this aspect of the invention relates to a pharmaceutical composition comprising a compound according to formula I, II or III, wherein R₁ to R₁₁ and R₂₁ to R₂₇ have the meaning as defined above and a pharmaceutically acceptable excipient for use in treating of a solid tumor characterized by decreased expression of NFKBIA.

20

In another aspect, the present invention relates to a method for determining if combination therapy with the cytostatic compound according to formula I, II or III and an alkylating agent is suitable for treating a patient with a solid tumor comprising the steps of

- a) determining the expression level of NFKBIA in a sample of tumor cells or tumor tissue
25 of the patient;
- b) comparing the determined expression level with a reference value;
- c) determining if the combination therapy with niclosamide and an alkylating agent is suitable for the patient based on the result of the comparison of step b), wherein underexpression or a deletion of NFKBIA indicates that the combination therapy is
30 suitable for the patient.

In another aspect, the present invention relates to a method for determining if therapy with the cytostatic compound according to formula I, II or III as defined above is suitable for treating a patient with a solid tumor comprising the steps of

- a) determining the expression level of NFKBIA in a sample of tumor cells or tumor tissue of the patient;
- b) comparing the determined expression level with a reference value;
- 5 c) determining if the therapy with niclosamide is suitable for the patient based on the result of the comparison of step b), wherein underexpression or a deletion of NFKBIA indicates that the combination therapy is suitable for the patient.

Preferably, the method of the invention is performed *in vitro*. Preferably, the patient suffers from a solid tumor as defined above. More preferably, the patient suffers from glioblastoma.

10

The sample is, preferably, a sample taken from the patient in question which comprises tumor cells or consists of tumor cells. Such samples can be taken e.g. by biopsy. A sample comprising other cells in addition to tumor cells may be pre-treated in order to increase the fraction of tumor cells in the sample.

15

Methods for determining the expression level of a gene are well known to the person skilled in the art. Gene expression can be determined by measuring the amount of mRNA-transcripts of the gene in question and it can also be determined by measuring the amount of the protein encoded by the gene. Preferred methods based on the measurement of the amount of mRNA-transcripts generated include quantitative real-time PCR and hybridization-based techniques such as microarrays. Preferred methods for the measurement of the amount of a specific protein include immunological methods such as the enzyme-linked immunosorbent assay (ELISA).

20

25 Underexpression is, preferably, defined by comparing the expression level determined in the sample in question with a specific method with the expression level determined in a reference sample or a group of reference samples with the same method. A preferred reference sample is a solid tumor with two intact copies of the gene encoding NFKBIA. Preferably, the promoter regions of both copies of the gene in said tumors do not comprise any mutations.

30

In a preferred embodiment of the present invention, underexpression of NFKBIA is caused by a heterozygous deletion of the NFKBIA locus (NFKBIA^{+/-}) at 14q13.

In another preferred embodiment of the invention, both copies of the gene encoding NFKBIA

are present, but at least one of these copies has an impaired function. A copy has an “impaired function” as understood in the present application if it does not encode a gene product, which has the same function and activity as the gene product encoded by the wildtype. NFKBIA has the ability to bind to nuclear factor kappa B (NF- κ B) in the cytosol. As long as it is bound to NF- κ B, NF- κ B cannot enter the nucleus and function as a regulator of transcription. Thus, the activity of NFKBIA which is most important in the context of the present invention is its binding to NF- κ B. Therefore, an “impaired function” as understood by the present application is, most preferably, impaired binding of NFKBIA to NF- κ B. This may be caused by exchanges of amino acids crucial for NFKBIA’s binding properties for other amino acids or it may be caused by deletions of parts of the gene encoding NFKBIA resulting in a shortened gene product.

A non-functional copy of NFKBIA may result the introduction of a stop-codon or a partial deletion. In this case a shortened gene product is produced which – if lacking crucial functional domains – has a decreased activity.

Non-functional copies may result from point mutations which cause exchanges of amino acids in the gene-product. If said exchange affects a part of the gene product which is important for its function, its activity is decreased or even abolished.

In another aspect, the present invention relates to a method for determining the molar ratio of niclosamide to temozolomide to be administered to a patient with a solid tumor comprising the steps of

- a) determining the expression level of NF κ BIA in a sample of tumor cells or tumor tissue of the patient;
- b) comparing the determined expression level with a reference value;
- c) determining the molar ratio of niclosamide to temozolomide based on the result of the comparison of step b), wherein
 - (i) an expression above the reference value indicates that the molar ratio shall be below 40 % niclosamide; and
 - (ii) an expression level below the reference value indicates that the molar ratio shall be equal to or larger than 40 % niclosamide.

All definitions given above in this application apply to this aspect of the invention as well.

Figure Legends

Fig. 1: Niclosamide effectively inhibits pGBMs cellular viability. **(A)** Pharmacodynamic analysis of 21 pGBMs (#'s indicated) at day 5 following niclosamide exposure (concentration indicated). Data as mean \pm SD of triplicates. **(B)** Spectrum of IC50 values representing the concentrations that decrease the metabolic activity to 50% of control levels. Data collected from three human non-tumor neural cell populations (hnNCs, see Methods), five commercially available glioma/GBM cell lines (see Methods), and the 21 pGBMs (see **(A)**; Table 3). The inset depicts IC50 data from additional pair-wise comparative experiments (symbol coded) on pGBMs derived from tumor center vs. periphery, from primary vs. recurrent disease, from *MGMT* promoter hypermethylated vs. unmethylated specimens, and from samples with *NFKBIA*^{+/+} vs. heterozygous *NFKBIA* deleted genotypes (*NFKBIA*^{+/-}). P-values (***) $p < 0.001$ were calculated from comparing hnNCs and GBM cell line data with pGBMs, respectively, using the 1-way ANOVA and Tukey's post hoc tests.

Fig. 2: Niclosamide has cytostatic and cytotoxic effects. **(A)** Cell cycle analysis at 24 hours post application exposing a strongly increasing G1 peak in the niclosamide (niclo)-treated sample. **(B)** CYCLIN D1 (CCND1)-western blot of cell extracts derived at this time point. **(C)** Cellular growth kinetics after a single exposure to niclosamide (1.5 μ M; squares) or DMSO (0.01%; rhombi) (pGBM #'s 046, 078, 106; mean data \pm SD). **(D)** Graph depicting frequency of avital, i.e. Annexin V⁺ and/or Hoechst 33258⁺ cells at 5 days after application of niclosamide (black bars) or 0.01% DMSO (white bars). Inset: mean data (n=5 pGBMs; **) $p < 0.01$. Note the lack of pro-apoptotic effects in the non-malignant human cell sample #155. Right inset: representative scatter plots (#046). **(E)** The scratch assay (n=2 pGBMs) was performed at day two following exposure of 125nM niclosamide (dotted line) or of 0.0025% DMSO (solid line). The graph exemplifies the time course of scratch closure for case #046 (mean \pm SD; triplicate analysis).

Fig. 3: Niclosamide decreases the tumor-initiating potential of pGBMs. **(A)** Neurosphere assay (n=4 pGBMs; mean \pm SD). The graph depicts the relative frequency of primary (1 $^{\circ}$), secondary (2 $^{\circ}$) and tertiary (3 $^{\circ}$) neurospheres from niclosamide pre-treated

pGBMs (single exposure). Data show a persistent decrease of sphere-forming cells (SFCs). **(B)** Long-term cell growth data after single application of 1.5 μ M niclosamide (niclo; dotted lines) vs. 0.01% DMSO (solid lines) (mean \pm SD of triplicate analysis). **(C)** Kaplan-Meier survival curves of xenografts. For experimentation, pGBM #046
 5 cells were pre-treated with niclosamide (dotted) or DMSO (solid). 10⁶ vital cells were collected at day 5 and stereotactically injected into the striatum of immunocompromised mice. Distressed animals were euthanized. With one exception (niclosamide; red dot), animals showed intracerebral tumor manifestation.

10 Fig. 4: Niclosamide affects several cancer regulating signaling pathways simultaneously. Western blot analysis of cleaved-NOTCH1 **(A)** and phospho-S6-protein **(B)** was performed at 5 days after single dose application. **(C)** Data quantification reveals a significant decrease of nuclear phospho-CTNNB1 (Ser552)⁺ cells (**p<0.001, triplicates, mean \pm SD). **(D)** qRT-PCR analysis of WNT/CTNNB1 target genes
 15 (**p<0.001, triplicates, mean \pm SD). Expression levels relative to the DMSO control. P-values calculated using 1-way ANOVA analysis with Bonferroni post-test. Note, all experiments conducted with pGBMs #'s 046, 078, 106, and 118.

Fig. 5: Deletion and expression level of *NFKBIA* predicts synergistic activity of niclosamide
 20 and TMZ. **(A)** Western blot analysis of pGBMs with *NFKBIA*^{+/-} genotype (purple) vs. *NFKBIA*^{+/+} samples (green). Levels of phospho-RELA (p65-NF- κ B) indicating pathway activity were determined 3 days after niclosamide exposure. **(B)** Quantification of mRNA levels in *NFKBIA*^{+/+} (046, 078, 138) vs. *NFKBIA*^{+/-} (081, 106, 066) pGBMs in response to niclosamide (light) or DMSO (dark) exposure. Data
 25 presented as relative to DMSO control. Inset depicting base-line mRNA expression levels of *NFKBIA*. **(C)** Combinatorial pharmacodynamics of TMZ and niclosamide in *NFKBIA*^{+/+} (n=4) vs. *NFKBIA*^{+/-} (n=3) pGBMs. Increasing concentrations of niclosamide were supplied either in combination with 50 μ M TMZ or with 0.05% DMSO as control. Data exemplified by #'s 046 (*NFKBIA*^{+/-}, square and triangle) and
 30 106, (*NFKBIA*^{+/+}, inverted triangle and diamond) and presented as mean \pm SD of triplicates. Arrows highlight synergistic activity for the *NFKBIA*^{+/-} sample. Note, data for the other investigated pGBMs are listed in Table 2.

Fig. 6: Niclosamide dose-response curves. The metabolic activity as a measure for cellular

viability was determined at 5 days after compound exposure (mean data \pm SD, triplicate analysis). Data relative to control (DMSO) levels. The IC50 was defined as concentration of niclosamide that reduced the metabolic activity to 50% control levels.

5 (A) Comparison of data for commercially available human glioma/GBM cell lines LN229, T89G, U87, U138, and U272 (grey) vs. 21 pGBMs (black; mean \pm SD, compare with Figure 2A). (B) Comparison of data for human non-malignant neural (control) cell samples PKI-3, #155, and H9.2 (grey) vs. 21 pGBMs (black; mean \pm SD, compare with Figure 2A). (C) Evaluation of paired samples derived from tumor core (squares) vs. tumor periphery (triangles) for pGBM #'s 046, 066, and 078. (D)

10 Evaluation of paired samples derived from primary disease (squares) vs. recurrent disease (triangles) for pGBM #'s 091, 118, and 132. (E) Comparison of data for *MGMT* promoter hypermethylated pGBM samples #023 and #025 (triangles) vs. *MGMT* promoter unmethylated pGBM samples #'s 046, 106, and 138 (squares). (F) Comparison of data for *NFKBIA*^{+/+} pGBM samples #'s 066, 081, and 106 (squares) vs.

15 *NFKBIA*^{+/-} pGBM samples #'s 046, 078, and 118 (triangles).

Fig. 7: Niclosamide induces a transient G1 phase arrest in pGBMs. Cell cycle analysis revealed similar results for cases #046 and #106 (PI, propidium-iodide). Shown is #046 exposed to 1.5 μ M niclosamide vs. 0.01% DMSO at (A) 6h, (B) 12h, (C) 24h,

20 (D) 48h, (E) 72h, and (F) 5d.

Fig. 8: Niclosamide depletes the tumor-initiating potential of pGBM #GNV019. (A) Long-term cell growth data after single dose niclosamide application (1 μ M, dotted) vs. DMSO (0.01%, solid). (B) Xenograft experiments were conducted similar to methods

25 described for Figure 4C, with the exception that neonatal mice were used as recipients. Kaplan-Meier survival curves depict the course of DMSO pre-treated (solid) vs. niclosamide (dotted) pre-treated cell grafts. Intracerebral tumor manifestation was noted in 8/9 animals from the DMSO control group. In contrast, no animal that received niclosamide pre-treated grafts showed evidence for tumor formation

30 (censored events). Calculation of the p-value based on the log-rank test.

Examples

Reagents

The reagents used in the experiments set out below can be freely purchased, specifically; alamarBlue[®], prodidiumiodide, and Hoechst33258 were purchased from Life Technologies; niclosamide and temozolomide were purchased from Sigma-Aldrich; FITC Apoptosis Detection Kit I were purchased from BD Bioscience.

5

Mice

The Ethical Committee of the University of Bonn, Medical Centre approved all studies involving animals. Rag2^{-/-}Il2rg^{-/-} mice were acquired from Taconic Farm Inc., contractor of the National Institute of Allergy and Infectious Diseases' investigators (42). SCID/Beige mice were purchased from Jackson Laboratory.

10

Tissue samples

Tumor tissue derived from GBM surgery and hippocampus tissue (case #155) derived from epilepsy surgery at the Department of Neurosurgery, University of Bonn Medical Centre. Patient characteristics are detailed in Table 3. pGBM case GNV019 derived from surgery of a 9-year-old boy at the University of Florida Department of Neurosurgery. The local Ethics committees at both sites approved the studies; all patients – or their guardians, provided informed consent. Tissue diagnosis and grading based on the current classification of the World Health Organization (43) and confirmed by two independent neuropathologists at the Department of Neuropathology, University of Bonn Medical Centre (the National Brain Cancer Reference Center).

15

20

25

Table 3

Patient data. List of patients and tissue specimens investigated in this study									
Patient	Diagnosis	Sex	Age	Histology	RPA Class	Primary therapy	PFS	OS	MGMTstatus
021	new	m	78	GBM	V	R, RT/TMZ, 2xTMZ (5/28)	4	12	unmet
023	new	f	79	GBM	V	RA	NA	9	meth
025	new	m	70	GBM	V	R, RTA	NA	NA	meth
035	new	f	75	GBM	IV	RB	1	1	unmet
046D	new	m	76	GBM	IV	RB	1	1	unmet
066D	new	f	69	GBM	IV	R, RT/TMZC	2	2	unmet
078D	new	m	52	GBM	IV	R, RT/TMZ, 2xTMZ (5/28)	5	10+	unmet
081	new	w	86	GBM	IV	RA	NA	17	unmet
091E	new	m	52	GBM	IV	R, RT/TMZ, 4xTMZ (5/28)	7	10	unmet
106	new	f	68	GBM	IV	R, RT/TMZ, 1xTMZ (5/28)	5	5+	unmet
116	new	f	67	GBM	IV	R, RT/TMZ	3	7	unmet
118E	new	m	63	GBM	IV	R, RT/TMZ, 4xTMZ (5/28)	7	9	unmet
132E	new	m	75	GBM	IV	R, RT/TMZ, 4xTMZ (5/28)	7		n.d.
135	new	m	41	GBM	IV	R, RT/TMZC	8	9	n.d.
138	new	w	54	GBM	IV	R, RT/TMZ, 5xTMZ (5/28)	10	14+	unmet

A: Patient denied further treatment; B: Postoperative complications; C: Discontinuation of therapy due to clinical deterioration; D: Two pGBM samples were derived from this patient, one from the tumor core (center) and the second from the tumor periphery (see (10)); E: Two pGBM samples were derived from this patient, one at the time of primary disease and the second at the time of disease recurrence; R: Tumor resection; RT: Standard radiotherapy; RT/TMZ: RT plus continuous daily temozolomide (concomitant); TMZ: Temozolomide (5/28: days 1 to 5 out of a 28-day-cycle) PFS: Progression-free survival; OS: Overall survival ; meth: methylated MGMT promoter; unmet: unmethylated MGMT promoter

Tissue handling and culture of primary cells

Handling of fresh biopsy samples and derivation of pGBMs (10) and hippocampus tissue-derived AHNPs (#155) (11) were performed as described recently. Media conditions for #GNV019 cells are detailed in (44). Media conditions for all other pGBM and AHNP samples are described in (8). Data were generated from culture passages 7 to 13.

Culture of established glioma/GBM cell lines

LN229, T98G, U87(MG), U138, and U373(MG) cells were maintained and analyzed in DMEM/F12-based 10% fetal calf serum (Hyclone)-supplemented adherent conditions.

Culture of neural stem cells from human ES and iPS cells

Together with primary AHNPs (see above), two human long-term self-renewing neural stem cell cultures (lt-NES) were used in this study as non-malignant neural control cells. The lt-NESs were originally derived from the human embryonic stem cell line H9.2 (45) and from the human induced pluripotent stem cell line PKa (46). Conditions for the maintenance of lt-NESs were recently described (45, 47).

Primary drug screening and pharmacodynamic analysis

The tested compounds were supplied to cells proliferating in a linear-exponential phase. For all used cell samples, respective titration experiments were conducted before analysis. 24 hours after seeding $2-3 \times 10^3$ cells/well into laminin/ poly-L-ornithine coated 96-well plates, cells were treated with $1 \mu\text{M}$ of each compound (stock solution 10mM in DMSO). Control cells were treated with 0.01-0.1% DMSO. Five days after application, metabolic activity as a measure of cell viability was determined using the alamarBlue[®] assay according to the manufacturers recommendations (Life Technologies). Fluorescence was measured using an Infinite200 microplate reader (Tecan) at $\lambda_{\text{ex}} = 540\text{nm}$ and $\lambda_{\text{em}} = 590\text{nm}$. Experiments were performed in triplicates for each sample.

For pharmacodynamic analysis, 5×10^4 cells were plated in 12-well-plates at 24 hours before application of compound-series and compound combinations, respectively. alamarBlue[®]-based analysis was conducted at 3-5 days post treatment. Experiments were performed in triplicates. IMC50 was defined as the compound concentration that reduced the metabolic activity by 50% compared to control conditions and determined via data analysis in GraphPad Prism 4.0.

Proliferation kinetics

Five days post treatment, 4.7×10^4 vital cells were plated into 3.5cm laminin/poly-L-ornithine coated plastic dishes, and four to six days later trypsinized, harvested, counted, and re-plated at a density of 4.7×10^4 . The procedure was repeated 4-5 times. For long-term monitoring of niclosamide-induced alterations to cellular growth, cell confluence was determined using the CellaVista[®] system Analyzer (Roche Diagnostics) according to the manufacturer's instructions

10 Cell migration analysis

5×10^4 cells were plated into 12-well-plates coated with laminin/poly-L-ornithine. Cells were treated with 125nM niclosamide every 24 hours for 4 days. Three days after plating (at a cell density of 70%), a scratch/wound was inflicted with a sterile pipette tip. Thereafter, culture media was exchanged to remove non-adherent cells. The Plaque Assay application of the CellaVista[®] system (Roche Diagnostics) was used according to the manufacturer's instructions to monitor the scratch/wound size over time. Triplicate analysis data \pm SEM.

Cell cycle analysis

Cells (5×10^4 per well) were grown in 12-well plates, and collected after treatment at times indicated. Cells were re-suspended in phosphate-buffered saline (PBS), fixed with ice-cold methanol and incubated for a minimum of 24 hours at 4°C. Cell pellets were collected by centrifugation and re-suspended in PBS solution, containing 50 μ g/ml propidium iodide and 50 μ g/ml RNase. Following incubation for 30 min at 37°C, cells were analyzed for DNA content using a FACS calibur flow cytometer (BD Bioscience).

25

Annexin V-based FACS analysis

1×10^5 cells were collected at 5 days following compound application, settled by centrifugation, re-suspended in 100 μ l AnnexinV buffer and incubated with 5 μ l Annexin V-FITC for 1 hour at room temperature. To distinguish between living and dead cells, labeling with 1.2 μ g/ml Hoechst 33258 was used. Annexin V presence was determined using standard conditions in a LSRII equipped with FACSDiva Software (BD Bioscience). 2×10^4 cells were counted per measurement. The term 'avital cells' was used for Annexin V⁻, Annexin V⁺/H33258⁺, and H33258⁺ cells.

30

Neurosphere assay

The neurosphere assay was performed to estimate the frequency of self-renewing clonogenic cells according to established protocols (10, 44). Neurospheres were quantified at 21 days in culture, triturated to a single cell suspension, and re-plated for analysis of the secondary and tertiary neurospheres. Multipotency was determined by plating a representative fraction of 3° neurospheres onto laminin/poly-L-ornithine coated glass coverslips allowing differentiation for 2-3 weeks before fixation in 4% paraformaldehyde (PFA).

Fluorescence analysis

10 Immunofluorescence analysis was performed on PFA-fixed samples according to standard protocols (44, 48) using antibodies against β III tubulin (Promega; monoclonal mouse, 1:1000), GFAP (DAKO, polyclonal rabbit, 1:600), β -catenin, and phospho- β -catenin (Ser552) antibody (both Cell signaling, 1:400). Cell nuclei were visualized with DAPI (Sigma).

Western blot analysis

15 Cell extracts were prepared at 24 to 144 hours following compound application and processed as described (49). Blot membranes were incubated overnight at 4°C with antibodies against Cyclin-D1 (1:1000; BD Pharmingen), cleaved-Notch1 (1:1000), or phospho-S6 protein (1:1000; all Cell signaling) respectively. After washing, peroxidase-coupled secondary antibodies (Santa Cruz) were added for 1 hour. After washing, blots were developed using the ECL system (Millipore). To confirm equal loading, blots were re-probed with an β -actin antibody (Sigma; 1:5000).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

25 Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Quantification of RNA concentration was performed with Nanodrop (Peqlab) and 400ng total RNA was reversely transcribed with oligo-dT primers in a reaction mix (1xRT-Puffer, 10mM DTT, 500 μ M pooled dNTPs, 1U/ μ l RNase inhibitor, 2.5U/ μ l Expand Reverse Transcriptase; all from Roche Diagnostics). Reaction occurred at 42°C for 1h. The cDNA product was amplified in a total volume of 10 μ l in 96well plates using the realplex 4 Mastercycler Epp Gradient S (Eppendorf) and the following PCR conditions: 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 30 seconds. For quantification, the following primers were used:

- S100A4 forward: 5'-CTCAGCGCTTCTTCTTTC-3' (SEQ ID NO: 1);
S100A4 reverse: 5'-GGGTCAGCAGCTCCTTTA-3' (SEQ ID NO: 2);
c-Myc forward: 5'-TTCGGGTAGTGGAA-AACCAG-3' (SEQ ID NO: 3);
c-Myc reverse: 5'-CAGCAGCTCGAATTTCTTCC-3' (SEQ ID NO: 4);
5 Cyclin D1 forward: 5'-CCGTCCATGCGGAAGATC-3' (SEQ ID NO: 5);
Cyclin D1 reverse: 5'-ATGGCCAGCGGGAAGAC-3' (SEQ ID NO: 6);
NFKBIA forward: 5'-ACACCAGGTCAGGATTTTGC-3' (SEQ ID NO: 7);
NFKBIA reverse: 5'-GCTGATGTCAATGCTCAGGA-3 (SEQ ID NO: 8).
- 10 For cDNA quantification of the house keeping gene glyceraldehyd-3-phosphat-
dehydrogenase (GAPDH) the following primers were used: forward: 5'-
TGCACCACCAACTGCTTAGC-3 (SEQ ID NO: 9); reverse:
5'-GGCATGGACTGTGGTCATGAG-3' (SEQ ID NO: 10). Data analysis was performed
with the Mastercycler Epp Realplex Software (Eppendorf). Mean values were calculated from
15 triplicate qRT-PCR reactions. Each mean value of the expressed gene was normalized to the
respective mean amount of the GAPDH cDNA.

Single nucleotide polymorphism array analysis (SNP)

- For evaluation of the NFKBIA locus whole-genome genotyping analysis was performed.
20 Genotyping of 299,140 SNPs was conducted using the Illumina HUMANCytoSNP-12 v2.1
according to the manufacturer's Infinium HD assay (Illumina, San Diego, USA). Data was
analyzed with Illumina GenomeStudio (2011.1) software including the Genotyping and
GenomeViewer modules. Chromosomal aberrations were identified by examination of LogR
ratios and B-allele frequencies.

25

MGMT Promoter Methylation Status

- The methylation status of the *MGMT* gene was determined by pyrosequencing as recently
described (50). In brief, 0.5 µg genomic DNA was treated with sodium bisulfite using the
EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturers
30 recommendations. For pyrosequencing, a 265 bp region was amplified from bisulfite
modified genomic DNA using primers MGMT-Py forward, 5'-biotin-
GGATATGTTGGGATAGTT-3' (SEQ ID NO: 11) (GenBank accession number AL355531,
nucleotides 46891 to 46908) and MGMT-Bis reverse, 5'-AAACTAAACAACACCTAAA-3'
(SEQ ID NO: 12) (GenBank accession number AL355531, nucleotides 47138 to 47156) with

biotin attached to the 5'-end of the forward primer. The primer used for the extension reaction was 5'-CCCAAACACTCACCAA-3' (SEQ ID NO: 13) which allowed sequencing of a 63 bp fragment containing 12 CpG sites. The pyrosequencing assay was designed to target CpG sites with strong methylation in GBM. Pyrosequencing was performed using PyroGold Reagents (Qiagen, Hilden, Germany) on the Pyromark Q24 instrument (Biotage, Uppsala, Sweden), according to the manufacturer's instructions. Pyrogram outputs were analyzed by the PyroMark Q24 software (Biotage, Uppsala, Sweden), using the CpG quantification software to determine the percentage of methylated versus unmethylated alleles according to percentage relative peak height. Tumor samples were scored methylated or unmethylated after measuring CpG methylation at individual positions and comparing with methylation data obtained from age matched normal brain tissues. Human reference DNA in vitro methylated by SssI methylase was used as positive control for methylation.

Tumor Xenograft experiments

Cells were harvested, counted and re-suspended in 0.1% DNase/PBS. Cell vitality was confirmed via trypan blue exclusion. For case #046, 10^6 DMSO-control (n=5)- or niclosamide (n=5)-pretreated pGBMs were injected stereotactically into the striatum of 12-week old Rag2Il2rg^{-/-} mice (0.8 mm anterior, 2 mm lateral, 3 mm deep). For case #GNV019, 2.5×10^4 sham control (Killer Plates[®] compound 2F05; n=9)- or niclosamide (n=6)-pretreated pGBMs were injected intracranially into P2 to P3 old Scid Beige mice. Mice were monitored daily and euthanized upon presentation with signs of distress/neurological symptoms or significant weight loss. The #019GNV experiment was terminated at day 169 with one remaining animal that did not appear distressed. For subsequent histological analysis, brains were removed, cryoprotected, and serially cut on a cryostat (Leica) at 20 μ m thickness. Every fifth section underwent routine H&E staining for histological analysis of tumor formation.

Statistical analysis

GraphPad Prism 4.0 software was used for statistical analysis. Data presented with error bars represent mean \pm SD from triplicate experiments unless otherwise noted. For pharmacodynamic analysis, p-values were calculated using the 1-way ANOVA and Tukey's post-hoc tests (Figure 1). For multiple comparisons, p-values were calculated using the 1-way ANOVA with Bonferroni post-hoc test (Figure 4E). If applicable, the two-sided Student's t test was used to determine statistical significance. A p-value of <0.05 was considered significant.

Results

Niclosamide is a previously unrecognized candidate for GBM therapy.

5 A library comprising of 160 synthetic and natural toxic substances was used for the screening approach. Four pGBMs, previously shown to maintain patient- and GBM-specific signatures and to contain sub-populations of tumor cells with and without stem cell qualities served as a discovery platform (#'s 023, 035, 046, 106; see (10)). Primary screening was conducted based on the alamarBlue[®] assay determining the metabolic activity as a measure for cellular

10 viability at day 5 following single application of the library's compounds. In the experiments, every compound that reduced the mean metabolic activity of the 4 pGBMs below 50% of control levels was considered as a 'hit'. 31 compounds fulfilled this criterion, amongst them niclosamide. Moreover, niclosamide indicated a sufficient potential to address inter-patient heterogeneity by impacting effectively on all four of the tested pGBMs (Table 1) and it

15 demonstrated a cancer-specific potential, as it did not appear to similarly affect hnNCs sample #155, a control case of non-malignant primary adult human neural progenitor cells (AHNPs; (11), Table 1). Niclosamide, revealed a selective pGBM-anticancer potential that had not yet been suggested for treating brain tumors. Niclosamide is known for decades and approved by many regulatory agencies as antihelminthic. Recent work in extra-neural, e.g. preclinical

20 colorectal cancer models suggested some activity of this drug (12, 13). However, given the scarcity of effective cytostatic compounds for the treatment of glioblastoma, its efficacy in this tumor entity was surprising..

Table 1

Metabolic activity of different cell lines after application of 1 mM niclosamide for 5 days (results of triplicate analysis), the metabolic inhibition of the cells is indicated in % of the activity of a control without niclosamide

pGBMs ¹				pGBMs average	Controls	
023	035	046	106		155 ²	U87 ³
32.3	5.6	34.6	33.0	26.4	75.4	66.5

¹primary glioblastoma cell lines used in this study

²non-malignant primary adult human neural progenitor cells

³a commonly investigated glioma cell line

Niclosamide is effectively and selectively inhibiting pGBMs cellular viability.

To validate the results obtained from primary screening of the library, pharmacodynamic
5 analysis was conducted using a formulation of niclosamide obtained from Sigma-Aldrich. A
total of 21 pGBMs were investigated, including the four cases already used in the screening
experiments. The obtained dose-response curves showed consistent courses for all samples
(Figure 1A). The concentrations at which niclosamide induced a 50% reduction of the relative
metabolic activity (IC₅₀) ranged from 300nM to 1.2µM. This contrasted to the more resistant
10 performance observed in reference- and control-cell samples. The IC₅₀ values of five
commonly investigated glioma/GBM cell lines (LN229, T98G, U87, U138, and U373, see
methods), here used as a reference, were calculated at 2.4 to 4.2-fold higher concentrations
(Figure 1B; Figure 6). The lower sensitivity of glioma/GBM cell lines to niclosamide
exposure may be due to their standard conditions of maintenance (i.e. serum-containing,
15 mitogen-free), a major factor that in the past might have interfered with many results of drug
screening at early developmental stages (7). Notably, however, analysis of the three non-
malignant hnNCs that were maintained in similar defined culture conditions as pGBMs also
revealed a significantly lower level of sensitivity (see methods; Figure 1B; Figure 6). This
suggested a pGBM-specific activity of niclosamide.

20 Considering the cellular and genetic diversity that characterizes GBM, we next investigated
niclosamide's pharmacological effect in pGBMs representing key clinical constellations (10,
14-16). Comparative experiments were therefore conducted with samples derived from (i) the
tumor core (center) vs. periphery region of the same GBM patient, (ii) primary vs. recurrent
disease of the same GBM patient, (iii) *MGMT*-promoter hypermethylated vs. unmethylated
25 tissue as well as from (iv) GBMs with heterozygous deleted *NFKBIA* vs. undeleted *NFKBIA*
genotypes (see below). The strong inhibitory activity of niclosamide could be demonstrated
similarly in all of these pGBM samples (inset Figure 1B; Figure 6). Together, these data
confirmed and validated our primary screening results, portraying niclosamide as a highly
effective and selective inhibitor of pGBMs.

30

Niclosamide has cytostatic, cytotoxic, and anti-migratory effects in pGBMs.

To further classify the inhibitory activity of niclosamide in pGBMs, studies on cell cycle,
vitality, and migratory function were conducted subsequent to a single exposure to the
compound. Propidium iodide (PI)-based flow cytometry analysis at revealed a transient G1

phase arrest of pGBMs peaking at 24 to 48 hours (Figure 2A; Figure 7). This coincided with a strong decrease of Cyclin D1 expression, a regulator of cell cycle transition from G1 to S phase (Figure 2B). Evidence for an immediate and transient cytostatic activity was similarly revealed upon examination of pGBM's growth kinetics (Figure 2C). With a resulting growth delay of 5 days, an additional cytotoxic response became apparent. At this time, pro-apoptotic effects of niclosamide caused a strong and pGBM-selective decrease of vital cells – as observed by phase contrast microscopy (not shown) and as quantified by Hoechst/Annexin V flow cytometric analysis (Figure 2D). Intriguingly, application of niclosamide at sub-toxic concentrations additionally caused anti-migratory effects on pGBMs, similar to recent findings described for colon cancer cells (12) (Figure 2E). Thus, niclosamide induced combined cytotoxic, cytostatic, and antimigratory effects in pGBMs.

Niclosamide inhibits pGBMs tumor-initiating activity.

In a next series of experiments it was aimed to determine the influence of niclosamide on the activity of tumor-initiating cells (TICs). TICs embody a severe functional consequence of intra-tumor heterogeneity as, at least in human GBM, it is anticipated that they are represented by a small subpopulation of stem-like, i.e. self-renewing and multipotent cells (e.g. (17-19)). However, their precise phenotypic characteristics remain elusive (20). We thus applied a combination of assays to measure their responses to niclosamide indirectly. First, the neurosphere assay (NSA) was used to estimate potential alterations to the pool of self-renewing and multipotent cells. In previous studies, we established their frequencies in the range from 0.25 to 1% among culture passage 5-10 pGBMs (see (10)). In the present study, three of these heterogeneous pGBM samples (#'s 046, 078, 106) were exposed to niclosamide, and vital cells were collected at day 5 for processing in the NSA (see methods). Quantification of primary, secondary and tertiary spheres from DMSO- vs. niclosamide pre-treated cells indicated that a single application of niclosamide reduced the frequencies of self-renewing, multipotent cells among pGBMs strongly (Figure 3A). Because niclosamide did not abolish the multipotent potential among the remaining self-renewing pGBMs at the applied concentration of 1.5 μ M (Figure 3A, inset), it was tempting to speculate that this setting could be used to similarly demonstrate a measurable reduction of tumorigenic cell frequencies *in vivo*. Parallel long-term growth analysis (CellaVista[®], cell confluence-based, see methods) of pGBMs indicated that at this concentration, the recovery of vital cells from cytostatic niclosamide effects had to be expected with a delay of 14-23 days (Figure 3B). Orthotopic xenotransplantation studies demonstrated, however, that animals engrafted with

niclosamide pre-treated vital cells survived considerably longer than expected (Figure 3C; Figure 8). The statistic significance of these results was paired with a lower extent to which tumor formation was observed in niclosamide pre-treated cell grafts. In one transplantational series (#GNV019), a single exposure to niclosamide completely extinguished the tumor forming capacity of pGBMs (Figure 8). In a second experimental series (#046), a strong reduction could be observed. Here, DMSO pre-treated #046 pGBMs that were grafted unilaterally into the striatum elicited severe signs of distress in recipient animals after 88 ± 5 d ($n=5$). Subsequent histological analysis revealed in every of these cases massive intracerebral tumor formation and a strong invasive capacity of engrafted cells along white matter tracts into the contra-lateral hemisphere. By contrast, the tumors that developed in 4/5 animals from niclosamide pre-treated #046 pGBMs at 153 ± 23 d after engraftment were smaller of size, with cells accumulating in areas adjacent to the striatal transplant site. Proliferative pGBMs were found clustered in the subventricular zone and dispersed throughout the corpus callosum (Figure 3E, inset), with individual cells reaching the contra-lateral hemisphere. This corresponded to early post-transplantational stages of DMSO pre-treated #046 cells. Apparently, the diffusely invasive nature of pGBMs sufficed during the long-term experiments to induce neurological dysfunction/distress in the animals that required their euthanization even before the manifestation of an expanding tumor mass. The combined data of our experiments, regardless, suggested strongly that already a single exposure of niclosamide did lead to an effective reduction of tumor initiating activity in pGBMs.

Niclosamide interferes with cancer-driving signaling cascades.

It is known that a circumscribed number of transcription factors and associated signaling pathways are overactive in human cancer cells (21). Evidence from previous studies had already suggested that niclosamide interfered with several of these in blood, breast, and colon cancer cells, specifically with Notch-, mTOR-, Wnt-/ β -catenin-, and NF- κ B- signaling (12, 13, 22-24). Hence, the study focused on this array of pathways for mode of action analysis in pGBMs. Cells were investigated at day 5 after a single-dose exposure to niclosamide ($n=4$ cases: #'s 046, 078, 81, 106). Western blots demonstrated a concentration-dependent inhibition of Notch pathway activity in the pGBMs, as indicated by decreasing levels of the cleaved Notch 1-protein (Figure 4A). Similarly, levels of the phosphorylated S6-protein as a major indicator of active mTOR signaling (25) could be shown to decrease in all samples (Figure 4B). Exploration of the Wnt-/ β -catenin pathway furthermore suggested a specific interference of niclosamide with the non-canonical (alternative) Akt-dependent regulation of

β -catenin's transcriptional activity. Characteristic for the active state of this mechanism, known to play an important role for tumor invasion, is an enhanced nuclear accumulation of β -catenin, phosphorylated at Ser⁵⁵² (26). The respective immunocytochemical exposure and quantification (Figure 5D) in pGBMs demonstrated a strong decrease of the nuclear phospho-
5 β -catenin (Ser552) antigen in response to application of niclosamide. Consequently, the expression of characteristic β -catenin target genes appeared significantly decreased in the pGBMs (Figure 4E) (12, 27, 28). Thus, niclosamide revealed a pleiotropic mode of action in pGBMs, inhibiting major cancer-driving signaling cascades simultaneously.

10 NFKBIA predicts synergistic effects of niclosamide and temozolomide.

In contrast to the consistent inhibitory impact on the Notch-, mTOR-, and Wnt-/ β -catenin-mediated pathways, niclosamide exhibited a variable effect on NF- κ B-signaling in pGBMs. Among the four cases used for mode of action analysis, Western blots revealed for only two (#046 and #078) a pathway inhibition as indicated by decreased levels of the phospho-p65-
15 NF κ B protein (Figure 5A). Subsequent genomic analysis (see methods) demonstrated for these two cases a heterozygous deletion of the *NFKBIA* locus (*NFKBIA*^{+/-}) at 14q13 that encodes for a major repressor of intracellular NF- κ B-signaling. As recent work had suggested that deletion and low expression of *NFKBIA* were associated with unfavorable clinical outcome in GBM patients (16), additional *NFKBIA*^{+/-} and *NFKBIA*^{+/+} pGBMs were identified
20 from the cohort for further investigation. These samples (n=3 for each group) revealed baseline expression levels that coincided with the respective genomic status of *NFKBIA* (inset Figure 5B). However, upon exposure to niclosamide, *NFKBIA*^{+/-} pGBMs were shown to strongly up-regulate their *NFKBIA* expression (Figure 5B). As similar responses were not observed in *NFKBIA*^{+/+} samples, the variable effects of niclosamide on NF- κ B-signaling in
25 pGBMs could be explained by a differential stimulation of *NFKBIA* expression in the *NFKBIA*^{+/-} samples. This observation intrigued, as it is known that down-regulation of *NFKBIA* in GBM cells is associated with a lack of response to alkylating agents, e.g. the standard GBM chemotherapeutic TMZ (29). On the other hand, it is known that the inhibition of NF- κ B alone may not severely affect most solid tumors, rather that it may help to prevent
30 resistance of cancer cells to chemotherapy (30, 31). Thus, a potential benefit that a combined application of niclosamide and TMZ might have in this setting was investigated.

Table 2

Combinatorial index evaluation for treatment with niclosamide plus TMZ in pGBMs indicates synergistic activity.

Case #	NFKBIA status	Expected survival proportion	Observed survival proportion	Combinatorial index
046	+/-	0.562	0.319	0.567
078	+/-	0.375	0.283	0.754
118	+/-	0.806	0.392	0.486
138	+/-	0.417	0.261	0.625
66	+/+	0.705	0.720	0.979
81	+/+	0.406	0.363	0.886
106	+/+	0.819	0.731	0.901

The combinatorial indices (CIs) for niclosamide and TMZ were expressed as ratio of observed vs. expected cell viability. Expected results were calculated as the proportion of viable cells following treatment with (only) 1 μ M niclosamide multiplied by the proportion of cells following treatment with (only) TMZ. (CI<1: synergy, CI=1: additive; CI>1:antagonism). *NFKBIA* status +/- (heterozygous deletion), +/+ (not deleted).

Experiments employed a cohort of 7 pGBMs (n=4, *NFKBIA*^{+/-}; n=3 *NFKBIA*^{+/+}; Table 2). All of these samples showed an unmethylated *MGMT* promoter status, a condition that indicates poor clinical responses to standard radio/TMZ-chemotherapy (2, 16). Combinatorial index analysis of niclosamide was conducted in the presence of 50 μ M TMZ. The concentration of TMZ was chosen based on the reported plasma peak levels in patients (32), which in many previous studies had shown to impact very little on the viability of glioma cells maintained *in vitro* (10, 33, 34). Similarly, we here observed that application of 50 μ M TMZ to the pGBMs reduced their metabolic activity to only 94 \pm 4% of control levels (n=7, triplicate analysis; data not shown). In combination with niclosamide, however, TMZ showed a particular effect on *NFKBIA*^{+/-} pGBM samples. Their dose-response curves showed a remarkable left-shift indicating stronger inhibitory activity of combined niclosamide/TMZ application compared to the *NFKBIA*^{+/+} samples (Figure 5C). Calculation of the combinatorial index (CI; (35)) suggested for *NFKBIA*^{+/+} pGBMs approximately additive effects (CI=0.92 \pm 0.05), and a clear synergistic activity of niclosamide/TMZ in all *NFKBIA*^{+/-} samples (CI=0.61 \pm 0.11) (Table 1).

These data suggested that niclosamide augments the anticancer effects of TMZ, the current GBM standard chemotherapeutic. Based on determining the genomic status of *NFKBIA* in GBM cells, a synergistic effect of niclosamide and TMZ may furthermore become predictable.

Discussion

The combined data of this study indicate that the pleiotropic anticancer effects of niclosamide are ideally suited to inhibit pGBMs from a variety of key clinical constellations. Cytostatic, cytotoxic, and anti-migratory effects are elicited, and the stem-like/tumorigenic cell fraction among pGBMs is strongly reduced. Thus, the issues of inter- and intra-patient tumor heterogeneity as well as the invasive nature of glial tumor cells that complicate any therapeutic approach in GBM (36) may become accessible by one drug. Several unique features of this compound nevertheless warrant future investigation for translation to brain tumor therapy.

Niclosamide is a common, by many regulatory agencies approved antihelminthic that has not yet been considered for the treatment of brain tumors. It is a salicylanilide, a chemical derivative of salicylic acid that was introduced by Bayer as a molluscicide in 1959. For medical use in animals and humans oral application is preferred causing only little toxicity. Studies in animals suggested no mutagenic, oncogenic, or embryotoxic activity and no cumulative effects. Its rate of absorption from the intestinal tract was estimated at 33% (for cumulative review, see (37)). The *in vitro* data suggest that niclosamide inhibits GBM core and periphery cells from primary disease, from disease recurrence, from *MGMT* promoter methylated and unmethylated, as well as from *NFKBIA*^{+/+} and *NFKBIA*^{+/-} GBM samples in concentration ranges that only marginally affect human non-malignant neural (control) cells.

For mode of action analysis, this study has mostly relied on evidence from previous work in the fields of hemato-oncology, colon, and breast cancer research (12, 13, 22-24). The findings of the present study have confirmed the results of these studies, exposing pleiotropic inhibitory effects of niclosamide on Wnt-/β-catenin-, Notch-, and mTor-signaling, which are known to play a pivotal role for GBM malignancy as well (16, 38-40). Of particular interest for future clinical application is niclosamide's hitherto unrecognized attribute to stimulate *NFKBIA* expression in *NFKBIA*^{+/-} cancer genotypes. While the responsible mechanism remains yet unclear, the resulting inhibition of NF-κB activity could be used to overcome resistance to

alkylating agents such as TMZ (29). The here demonstrated synergistic activity of niclosamide with the current standard GBM chemotherapeutic TMZ in *NFKBIA*^{+/-} pGBMs provides evidence for this assumption. It is highly probable that other cancer entities presenting with specific single-nucleotide polymorphisms and haplotypes of *NFKBIA*, e.g. Hodgkin's lymphoma, colorectal cancer, melanoma, hepatocellular carcinoma, breast cancer, and multiple myeloma (for collective references, see (16)) might profit from combining niclosamide with alkylating chemotherapeutic regimens.

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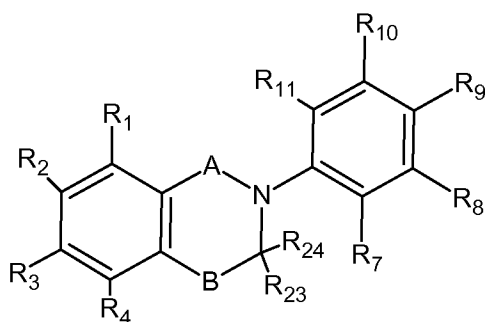
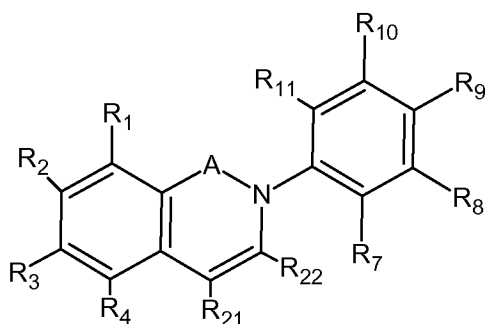
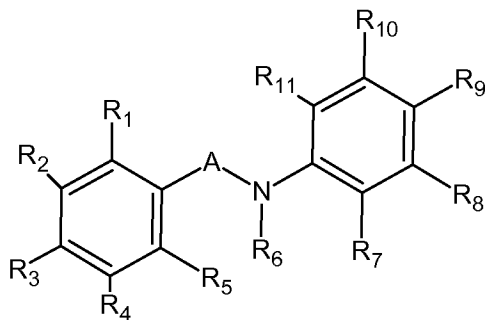
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Claims

1. A cytostatic compound according to formula I, II or III



wherein

A is carbonyl, methylene, hydroxymethinyl, alkoxymethinyl, aminomethinyl, oxime,
15 hydrazone, arylhydrazone, or semicarbazone;

B if present is $CR_{25}R_{26}$, O, S or NR_{27} ;

R_1 , R_3 , R_4 , R_8 , R_{10} , and R_{11} are independently hydrogen, hydroxyl, alkoxy, halogen or
 C_1 to C_6 alkyl;

R_2 and R_7 are independently halogen, hydroxyl or hydrogen,

R₅ if present is hydroxyl, phosphate, hydrogen, halogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, alkoxy, alkylthio or amino;

R₆ if present is hydrogen or C₁ to C₆ alkyl;

R₉ is nitro, hydrogen, hydroxyl, amino, halogen, alkyl, alkenyl, alkynyl, or aryl; and

5 R₂₁, R₂₂, R₂₃, and R₂₄, if present are independently hydrogen, hydroxyl, halogen, alkyl, alkenyl, alkynyl, cycloalkyl or aryl;

R₂₅ and R₂₆ if present are independently hydrogen, hydroxyl, halogen, alkyl, alkenyl, alkynyl, cycloalkyl or aryl; and

R₂₇ if present is hydrogen or C₁ to C₆ alkyl:

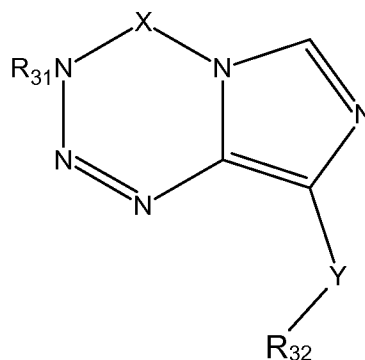
10 or salt thereof;

and an alkylating compound for use in treating of a solid tumor.

2. The compounds of claim 1, wherein the alkylating agent is an O⁶-alkylating agent.

15

3. The compounds of claim 1 or 2, wherein the alkylating compound has a structure according to formula IV



20

(IV)

wherein

25

X and Y are independently carbonyl, methylene, hydroxymethyl, alkoxyethyl, aminomethyl, oxime, hydrazone, arylhydrazone or semicarbazone,

R₃₁ is alkyl, hydrogen, alkoxy, alkenyl, alkynyl, cycloalkyl or aryl; and

R₃₂ is amino, hydrogen, hydroxyl or halogen

or salt thereof.

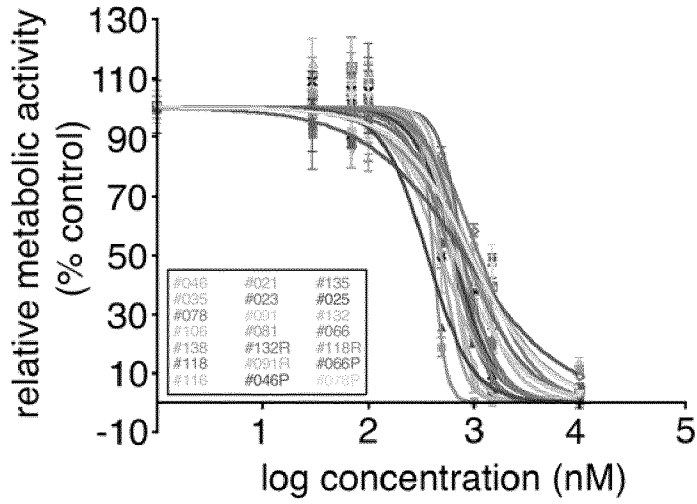
4. The compounds of any one of claims 1 to 3, wherein the cytostatic compound is niclosamide.
- 5
5. The compounds of any one of claims 1 to 4, wherein the alkylating compound is temozolomide.
6. The compounds of claim 4, wherein the molar ratio between niclosamide and temozolomide is in the range of 10 % niclosamide/90 % temozolomide to 90 % niclosamide/90 % temozolomide.
- 10
7. The compounds of any one of claims 1 to 6, wherein the solid tumor is glioblastoma.
- 15
8. The compounds of claim 7, wherein the glioblastoma is primary glioblastoma, de novo glioblastoma; secondary glioblastoma; recurrent glioblastoma, glioblastoma with increased methylation of the promoter of the gene O6-Methylguanin-Methyltransferase (MGMT), glioblastoma without increased methylation of the promoter of MGMT, glioblastoma with mutated p53, glioblastoma without mutated p53, glioblastoma with alterations of the gene encoding kappa light polypeptide gene enhancer in B-cells inhibitor (NFκBIA), glioblastoma without alterations of the gene encoding NFκBIA, glioblastoma with alterations of the gene encoding epidermal growth factor receptor (EGFR), glioblastoma without alterations of the gene encoding EGFR, glioblastoma with alterations of the gene encoding platelet-derived growth factor receptor (PDGFRA), glioblastoma without alterations of the gene encoding PDGFRA, glioblastoma with alterations of the gene encoding isocitrate dehydrogenase 1 (IDHI), glioblastoma without alterations of the gene encoding IDHI, glioblastoma with alterations of the gene encoding neurofibromatosis type 1 (NF1) and glioblastoma without alterations of the gene encoding NF1.
- 20
- 25
- 30
9. The compounds of any of claims 1 to 8, which are formulated for simultaneous or subsequent administration.
10. The compounds of claim 9, wherein the formulation for simultaneous administration is

a mixture of the two compounds.

11. The compounds of claims 1 to 10, wherein the cytostatic compound is formulated for resorption into the central nervous system.
- 5
12. A pharmaceutical composition comprising the compounds of any of claims 1 to 11 and a pharmaceutically acceptable excipient for use in treating of a solid tumor.
13. A compound according to formula I, II or III, wherein R_1 to R_{11} and R_{21} to R_{27} have the meaning as defined above for use in treating of a solid tumor characterized by a decreased expression level of NF κ BIA.
- 10
14. A method for determining if therapy with the cytostatic compound according to formula I, II or III as defined in claim 1 is suitable for treating a patient with a solid tumor comprising the steps of
- 15
- a) determining the expression level of NF κ BIA in a sample of tumor cells or tumor tissue of the patient;
- b) comparing the determined expression level with a reference value;
- c) determining if the therapy with niclosamide is suitable for the patient based on the result of the comparison of step b), wherein underexpression or a deletion of NF κ BIA indicates that the combination therapy is suitable for the patient.
- 20
15. A method for determining the molar ratio of niclosamide to temozolomide to be administered to a patient with a solid tumor comprising the steps of
- 25
- a) determining the expression level of NF κ BIA in a sample of tumor cells or tumor tissue of the patient;
- b) comparing the determined expression level with a reference value;
- c) determining the molar ratio of niclosamide to temozolomide based on the result of the comparison of step b), wherein
- 30
- (i) an expression above the reference value indicates that the molar ratio shall be below 40 % niclosamide; and
- (ii) an expression level below the reference value indicates that the molar ratio shall be equal to or larger than 40 % niclosamide.

Fig. 1

A



B

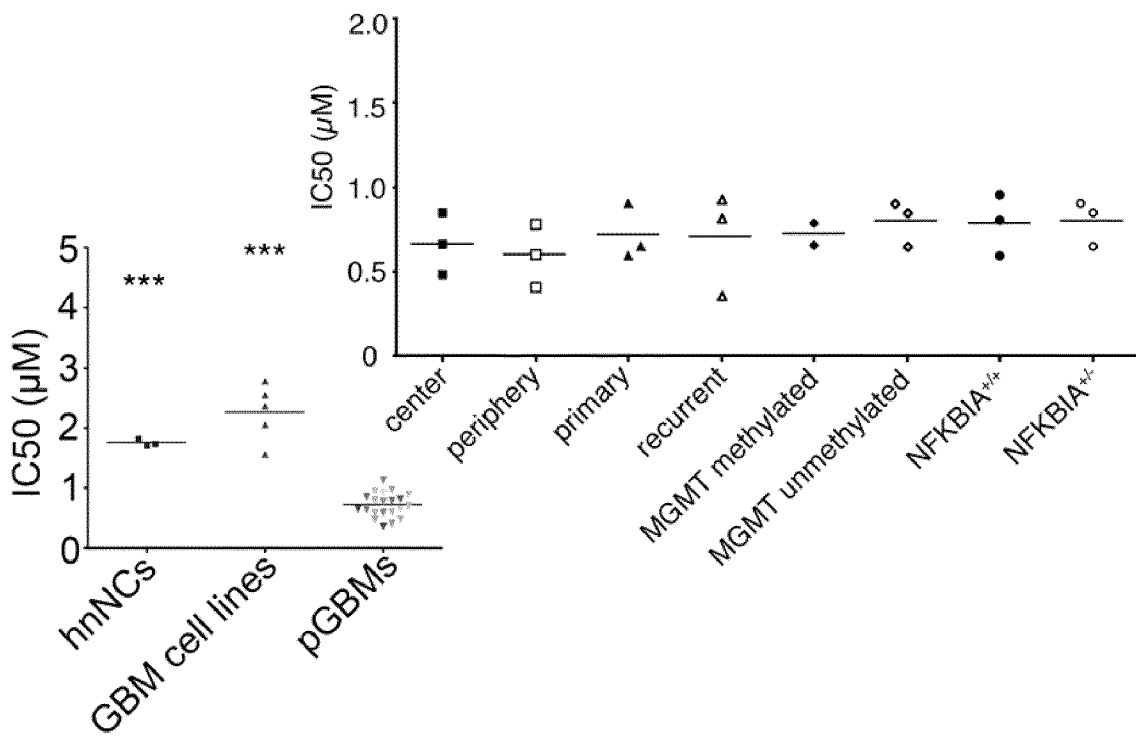
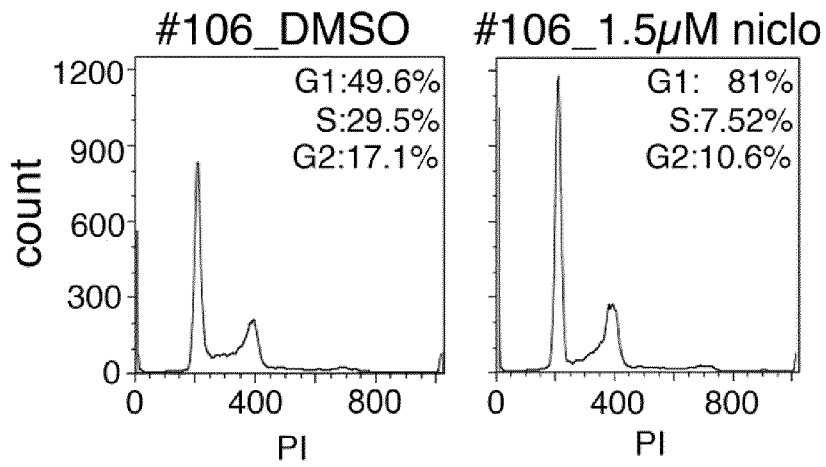
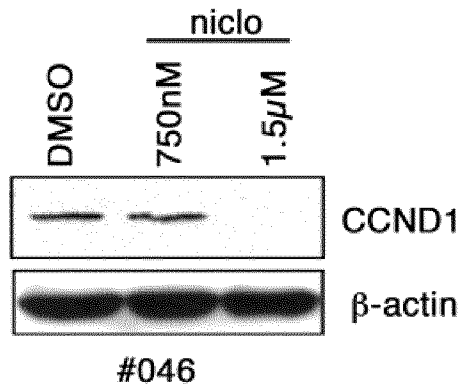


Fig. 2

A



B



C

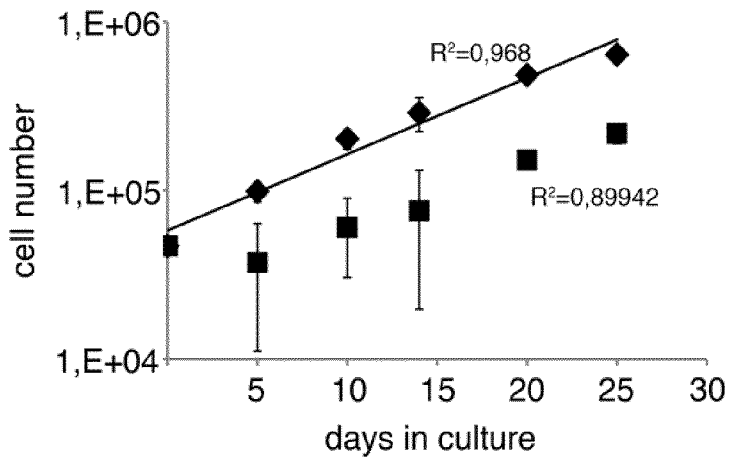


Fig. 2 (continued)

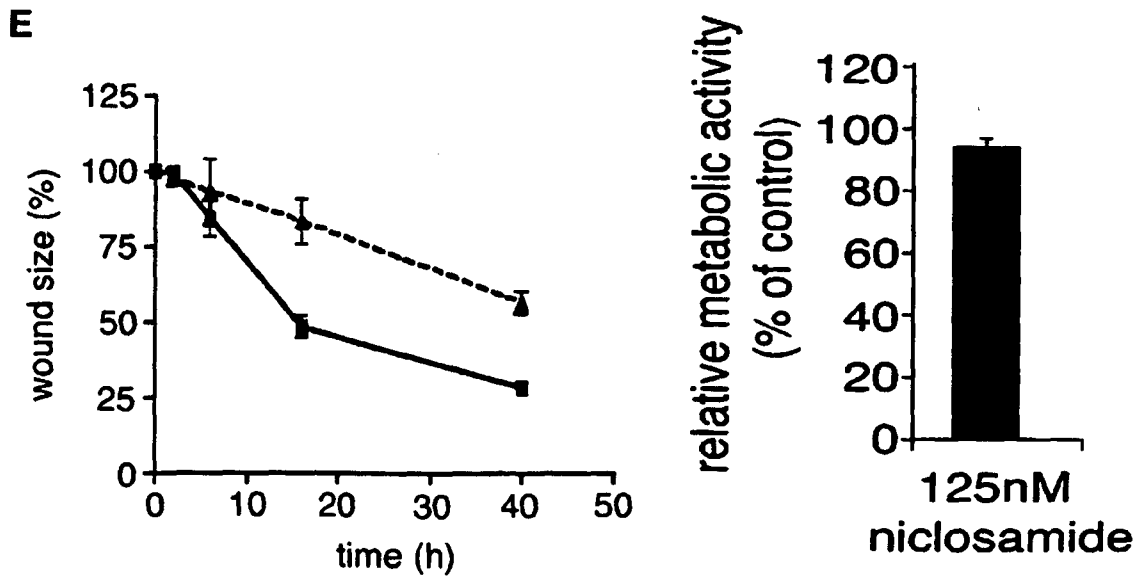
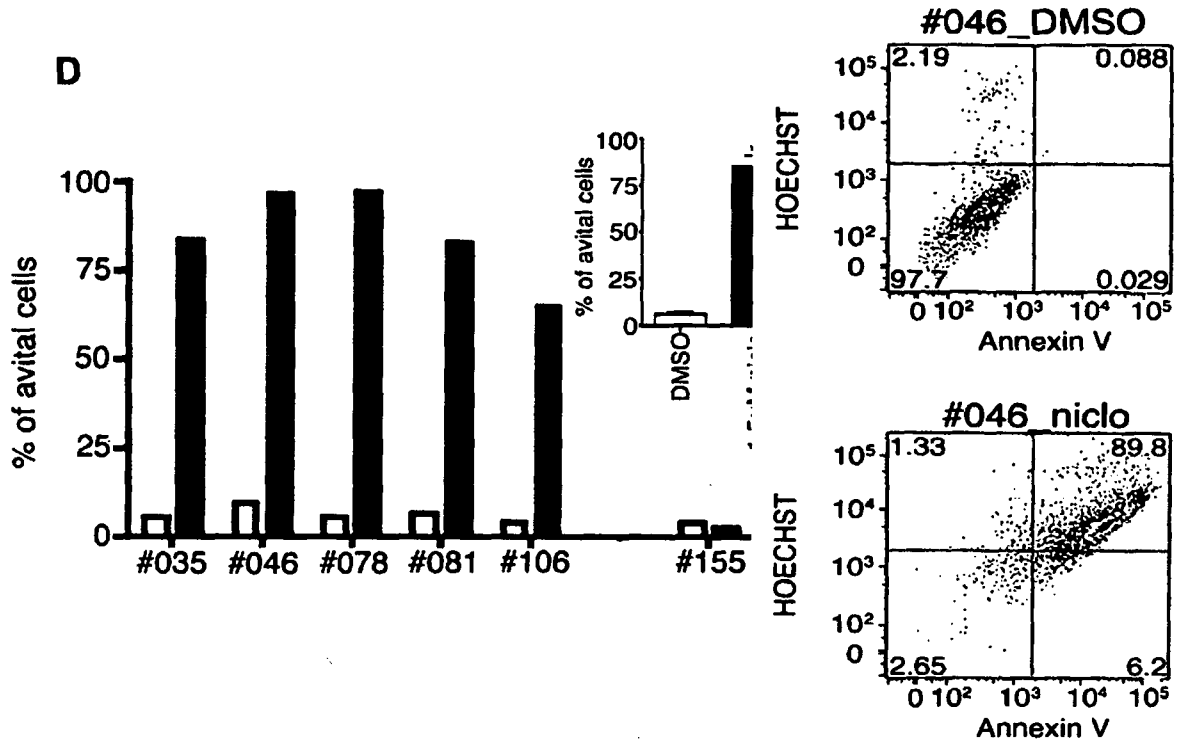


Fig. 3

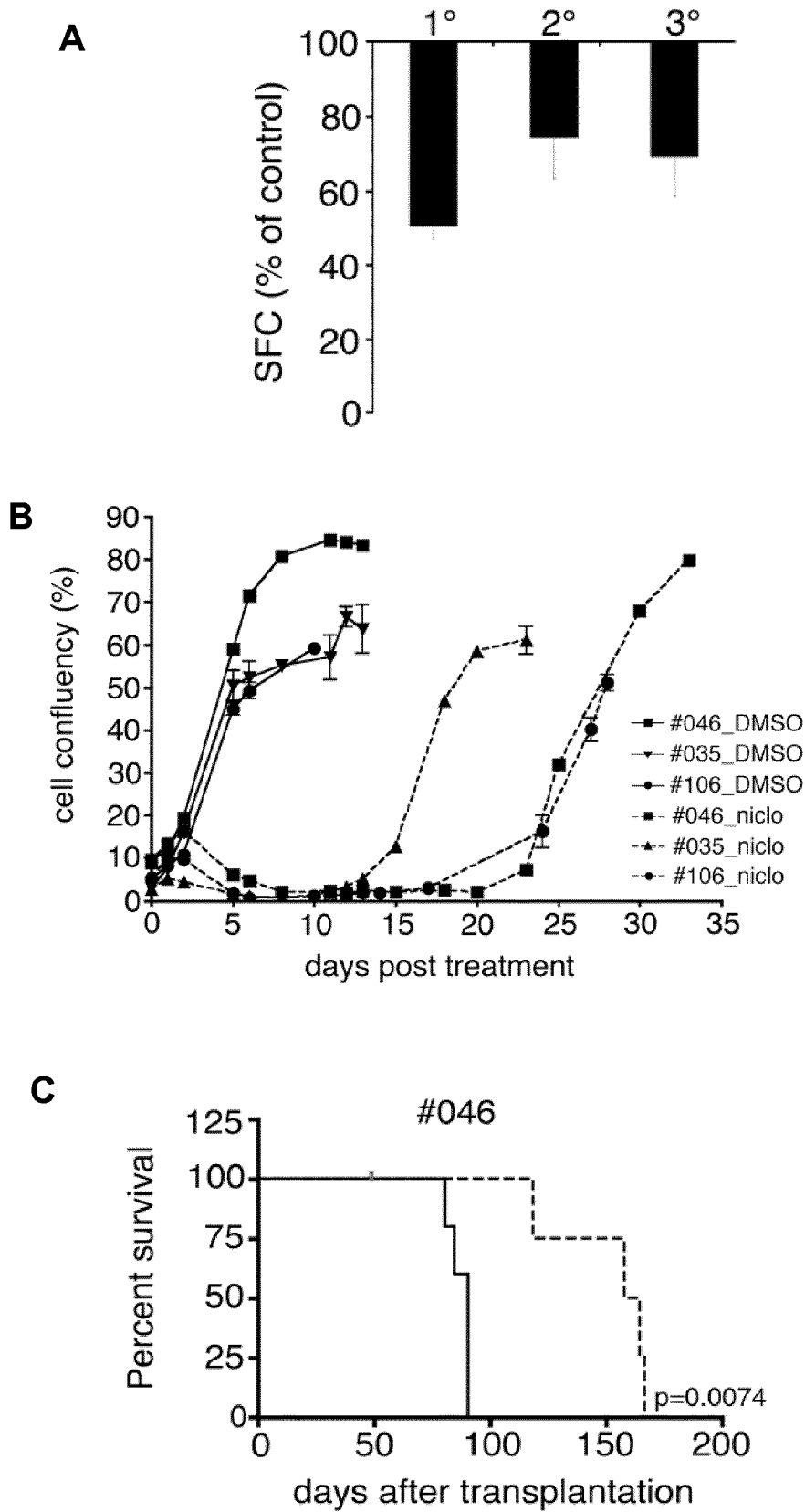


Fig. 4

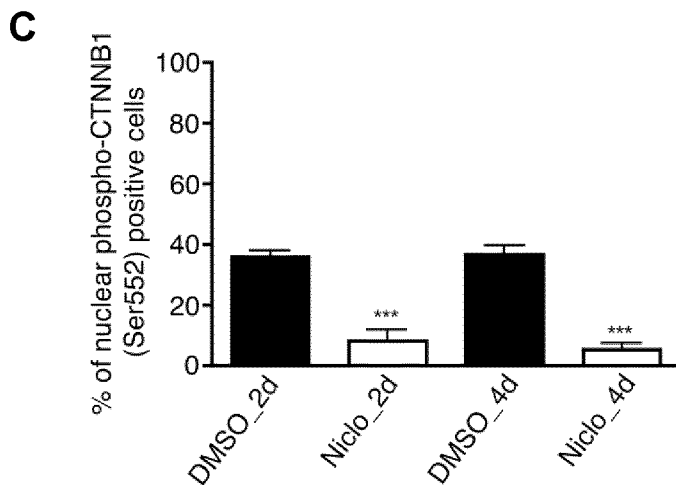
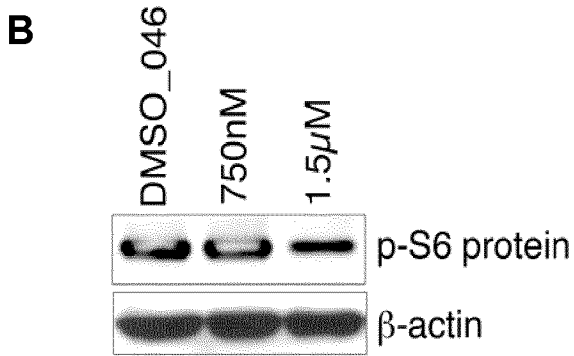
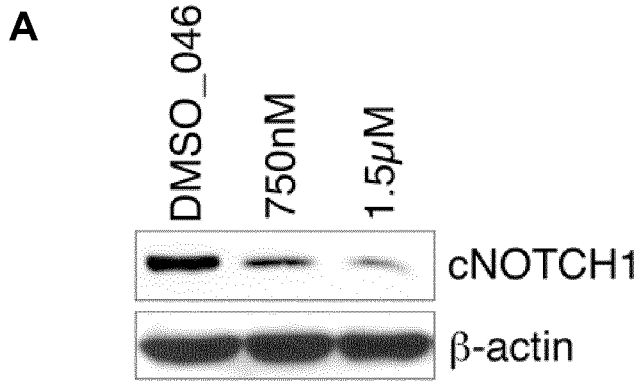


Fig. 4 (continued)

D

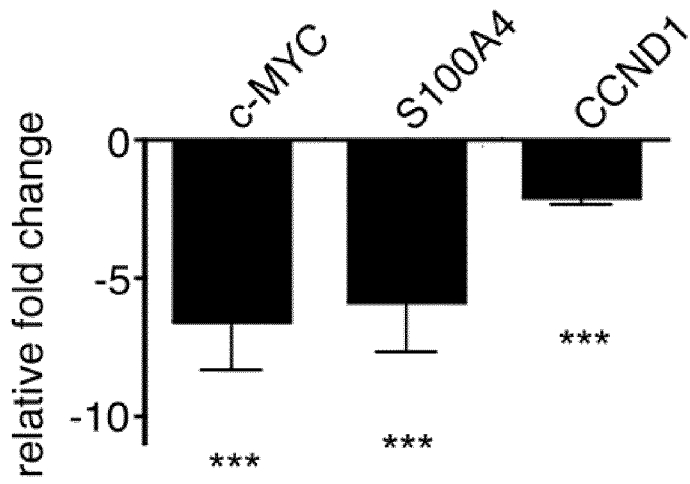


Fig. 5

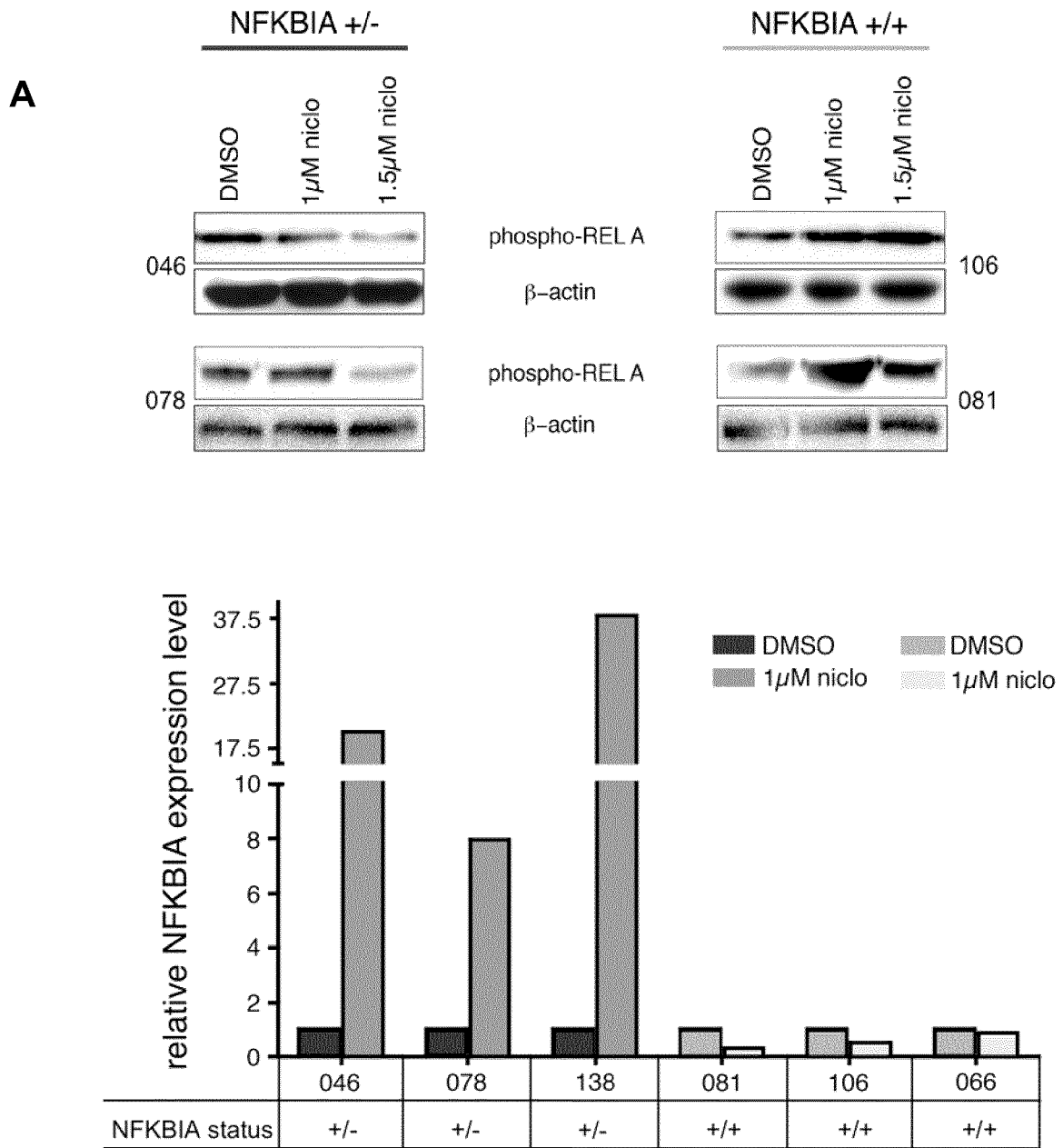


Fig. 5 (continued)

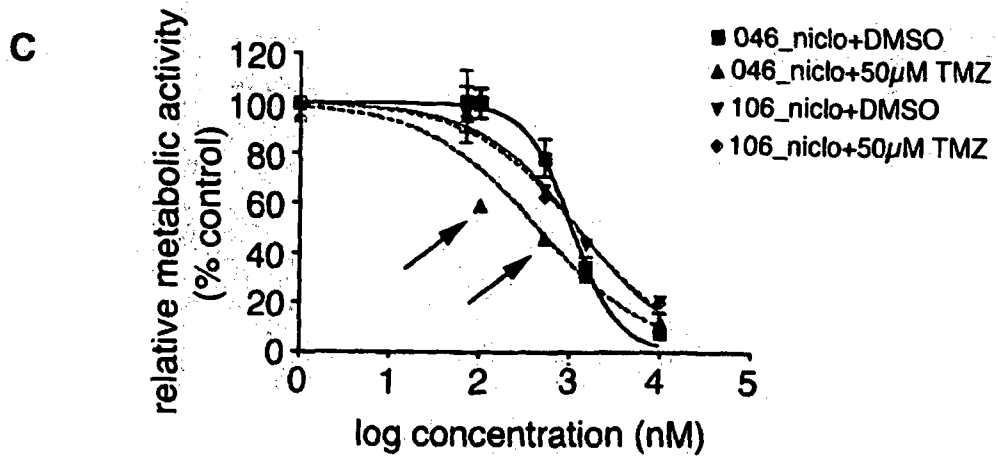


Fig. 6

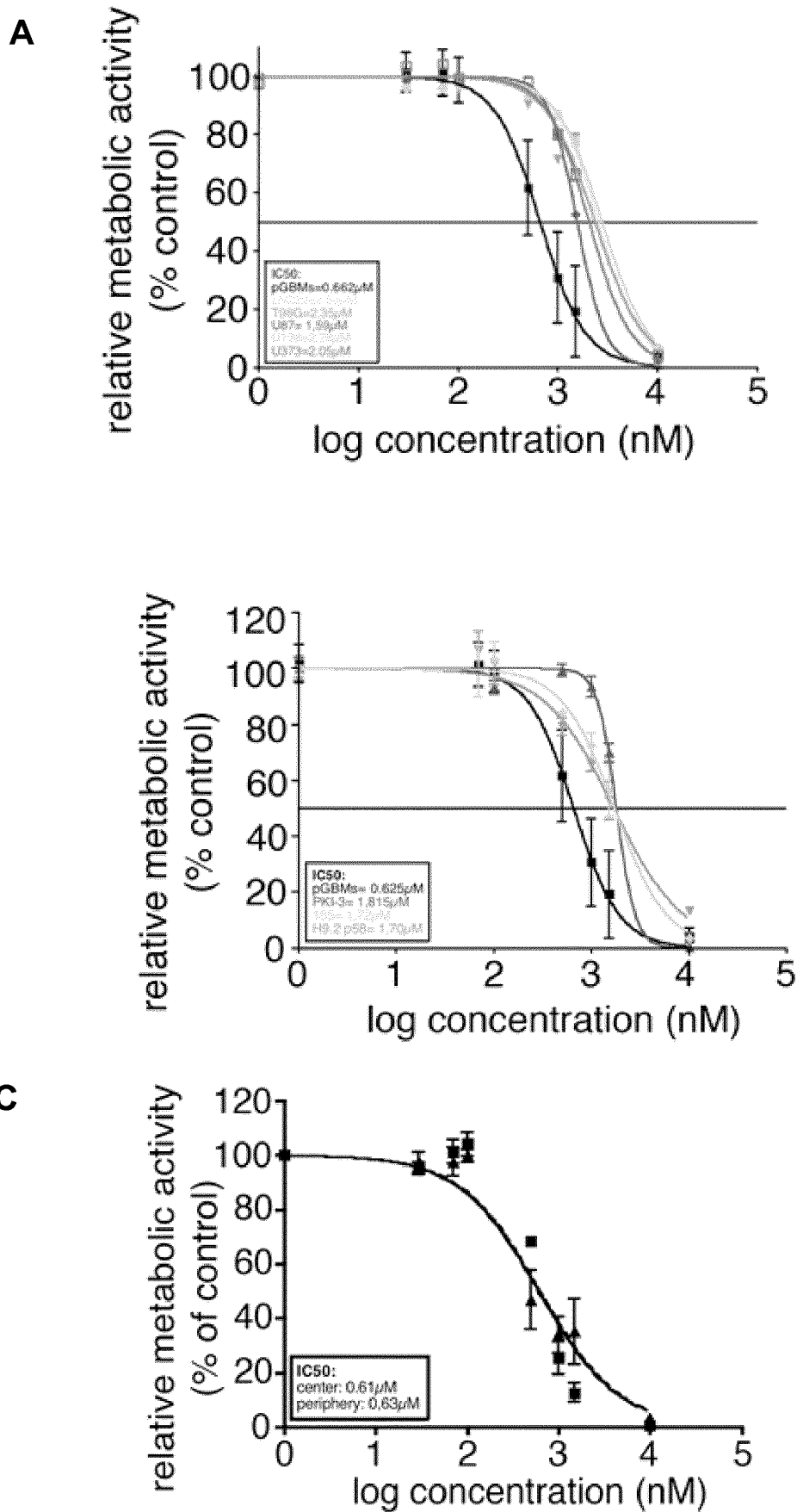


Fig. 6 (continued)

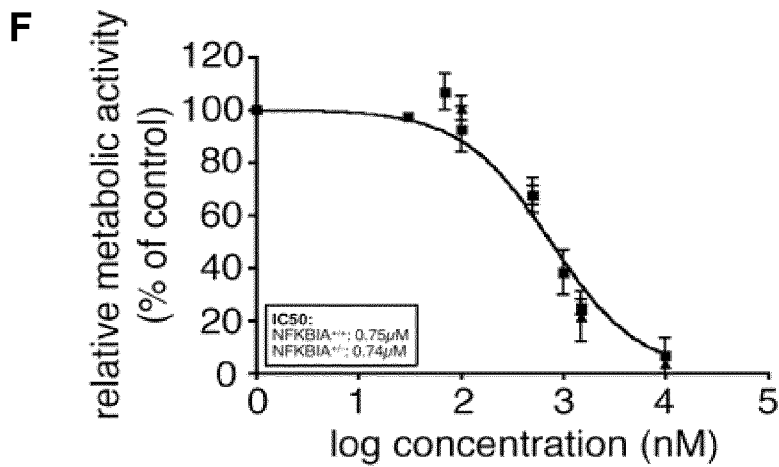
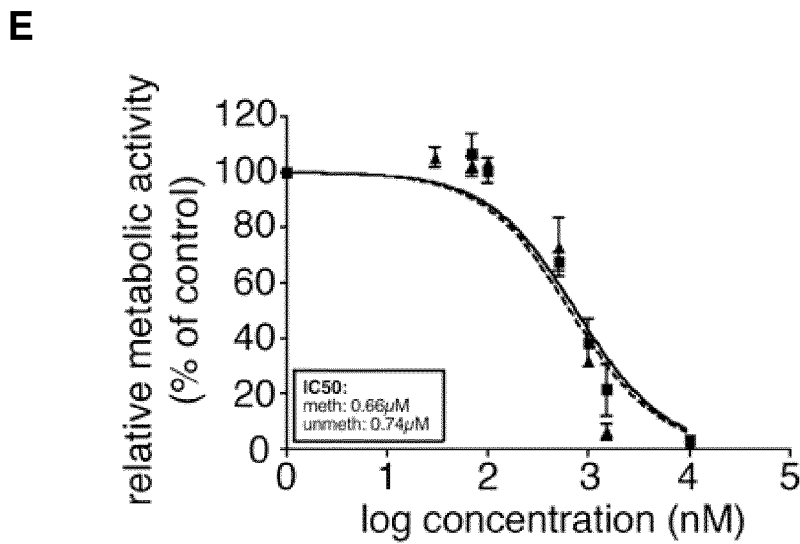
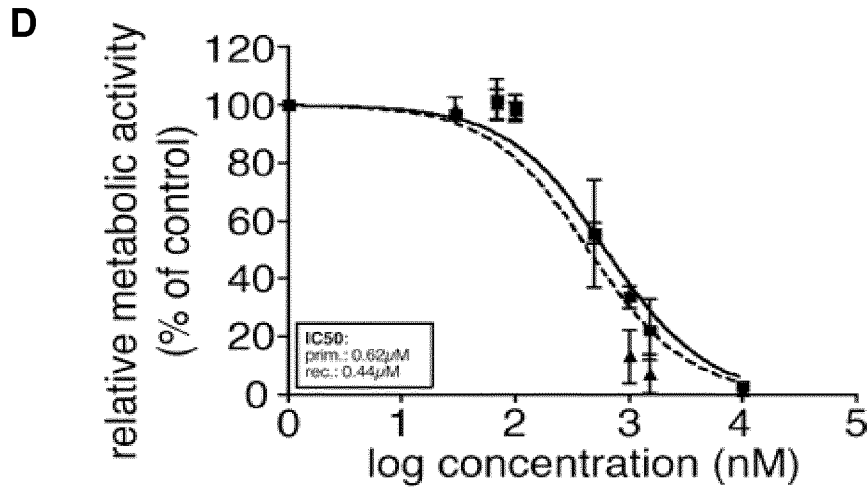
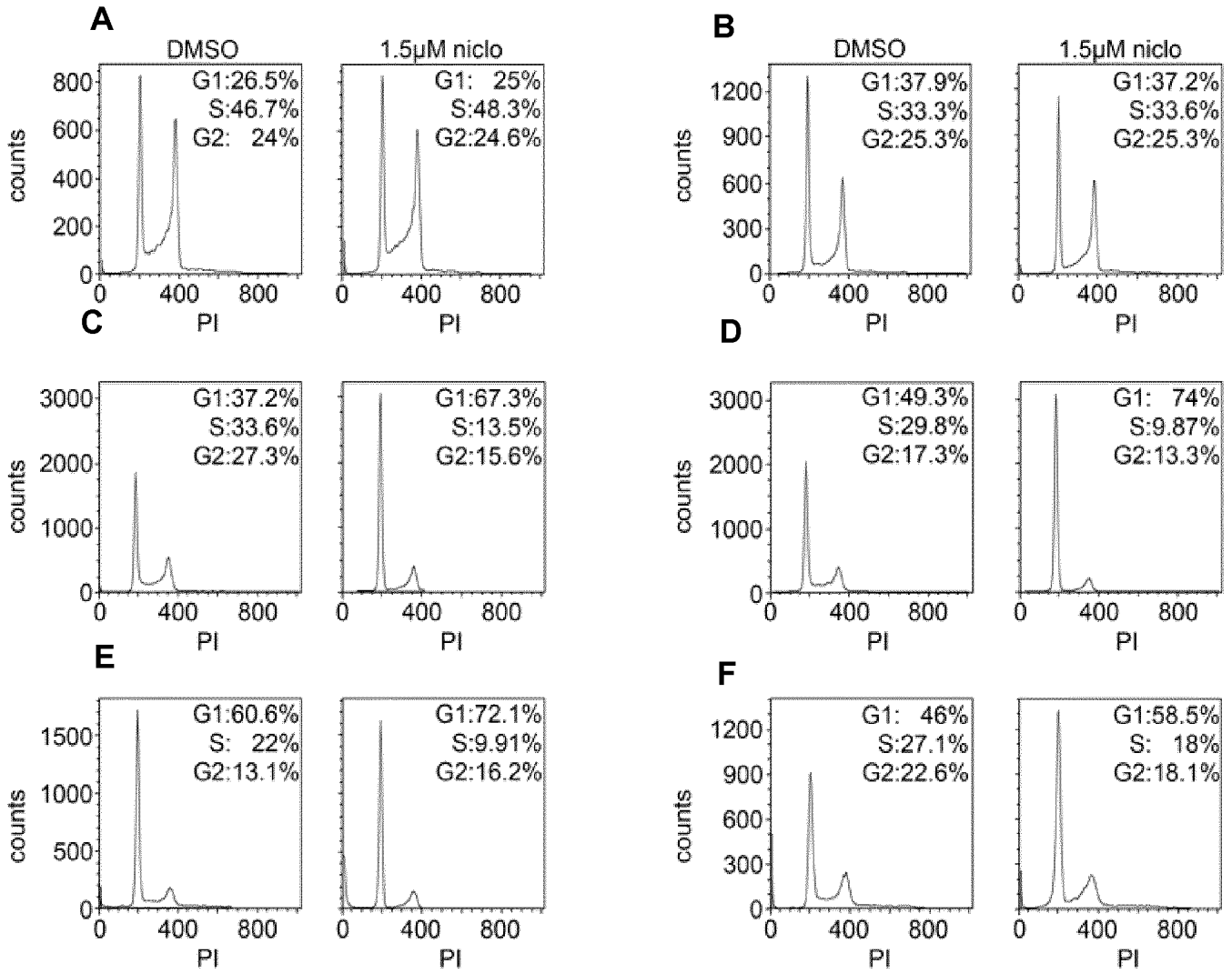


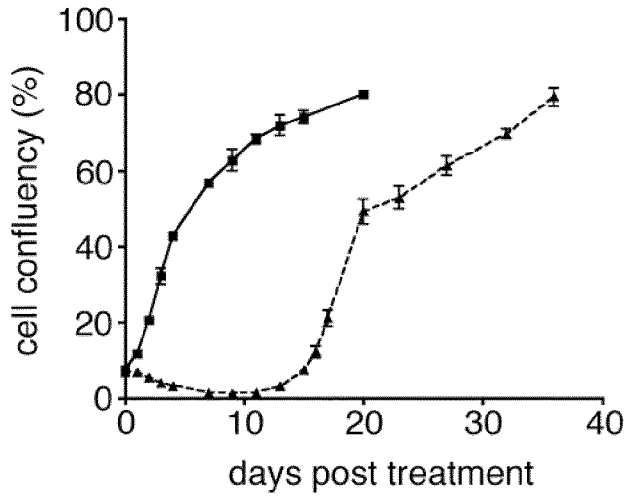
Fig. 7



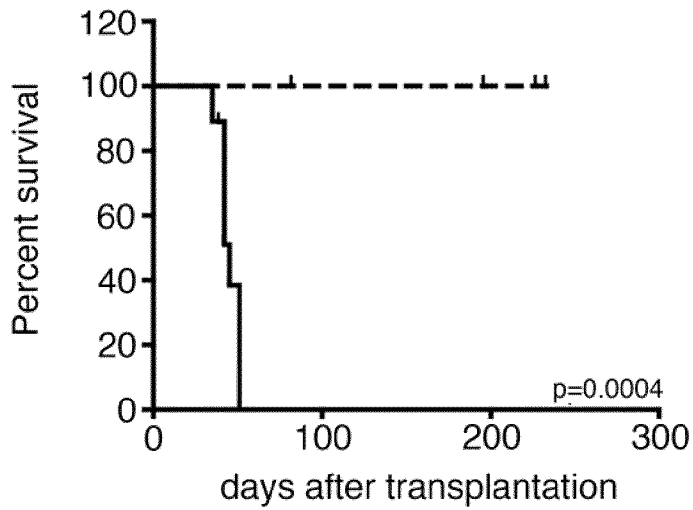
12/12

Fig. 8

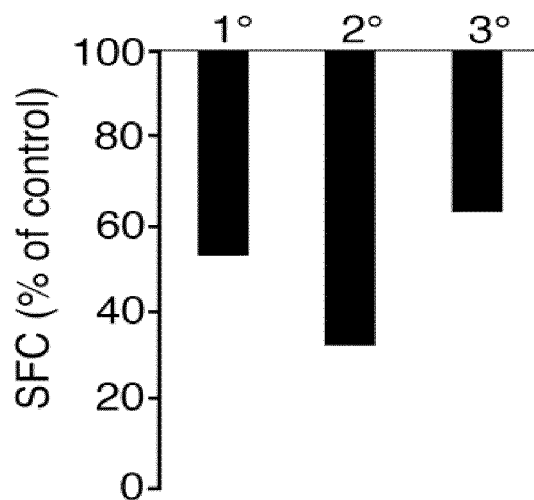
A



B



C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/065364

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

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

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

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

see additional sheet

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

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

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

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

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/065364

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K31/165 A61K31/4188 A61P35/00 A61K45/06 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) A61K A61P		
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, WPI Data, BIOSIS, CHEM ABS Data, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 2012/172069 A1 (LIFE AND BRAIN GMBH [DE]; SCHEFFLER BJOERN [DE]; GLAS MARTIN [DE]; SIM) 20 December 2012 (2012-12-20) page 22, line 24 - page 24, line 13 page 16, line 1 - page 17, line 17 page 35, line 3 claims 3,12	1-15
X	----- WO 2004/006906 A2 (COMBINATORX INC [US]; LEE MARGARET S [US]; KEITH CURTIS [US]; AUSPITZ) 22 January 2004 (2004-01-22) claims 1, 10-11 sentence 7 - sentence 19 page 7; table 1 examples 1-7 ----- -/--	1-15
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance	"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	
"E" earlier application or patent but published on or after the international filing date	"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	
"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)	"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	
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 6 June 2013	Date of mailing of the international search report 13/06/2013	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Renard, Delphine	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/065364

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/060951 A2 (BIONAUT PHARMACEUTICALS [US]; KHODADOUST MEHRAN [US]) 7 July 2005 (2005-07-07) claims 6,7 page 6, line 9 - line 30 -----	1-5,9-15
X	WO 2006/097323 A1 (WEBER LUTZ [DE]) 21 September 2006 (2006-09-21) claims 1, 7, 10, 13 -----	1-3,5, 7-14
Y	WO 02/46164 A1 (ASTRAZENECA AB [SE]; BARLAAM BERNARD [FR]; DANTZMAN CATHY [US]) 13 June 2002 (2002-06-13) page 16; compounds 1-5, 13 -----	1-3,7-14
Y	EP 1 454 898 A1 (AJINOMOTO KK [JP]) 8 September 2004 (2004-09-08) compounds 70,71 -----	1-3,7-14
Y	WO 2010/078916 A1 (PHILOGEN SPA [IT]; PEDRETTI MARTA [CH]; NERI DARIO [CH]) 15 July 2010 (2010-07-15) claims -----	1-15
Y	WO 2009/148623 A2 (STC UNM [US]; THOMPSON TODD A [US]; MACKENZIE DEBRA [US]; OPREA TUDOR) 10 December 2009 (2009-12-10) claims 6,10,11,12,18,19,20 -----	1-15
Y	US 2006/009506 A1 (WESTWICK JOHN K [US] ET AL) 12 January 2006 (2006-01-12) claims -----	1-15
Y	WO 2006/122007 A1 (COMBINATORX INC [US]; JOHANSEN LISA M [US]; LEE MARGARET S [US]; NICH0) 16 November 2006 (2006-11-16) page 3 - page 4; tables 1,2 claim 1 -----	1-15
E	WO 2013/049045 A1 (BIOMED VALLEY DISCOVERIES INC [US]; HALSE REZA [US]; SAHA SAURABH [US]) 4 April 2013 (2013-04-04) claims 1, 24, 39 examples 4-5 -----	1-3,5, 7-14
E	WO 2013/019469 A1 (WISTAR INST [US]; UNIV NORTH CAROLINA STATE [US]; HUANG QIHONG [US]; D) 7 February 2013 (2013-02-07) page 110; compound mir347 paragraph [0047] -----	1-3,5, 7-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/065364

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012172069	A1	20-12-2012	NONE
WO 2004006906	A2	22-01-2004	AU 2003249244 A1 WO 2004006906 A2
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 4, 6, 15(completely); 1-3, 5, 7-14(partially)

A compound according to formula (I) and an alkylating compound for use in treating of a solid tumor.

2. claims: 1-3, 5, 7-14(all partially)

A compound according to formulae (II) and (III) and an alkylating compound for use in treating of a solid tumor.
