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(54) **Title:** AMINOHETEROARYL COMPOUNDS AS MTH1 INHIBITORS

(57) **Abstract:** he present invention relates to an (S)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing cancer in a subject. The invention further relates to a pharmaceutical composition comprising said compound. Another aspect of the invention is directed to an in vitro method for determining the effectiveness of said (S)-enantiomer of an aminoheteroaryl compound, or said pharmaceutical composition, the method comprising the steps of: (a) obtaining a cell or tissue sample from a subject; and (b) determining the subject's NUDT1/MTH1 -status; wherein a NUDT1/MTH1-positive cell or tissue sample is indicative of an effective treatment and/or prevention of cancer. In addition, provided herein is a screening method for identifying a target of an (S)-enantiomer of an aminoheteroaryl compound. Furthermore, in context of this invention, the herein described compounds inhibit the biological activity of MTH1.



Aminoheteroaryl compounds as MTH1 inhibitors

The present invention relates to an (*S*)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing cancer in a subject. The invention further relates to a pharmaceutical composition comprising said compound. Another aspect of the invention is directed to an *in vitro* method for determining the effectiveness of said (*S*)-enantiomer of an aminoheteroaryl compound, or said pharmaceutical composition, the method comprising the steps of: (a) obtaining a cell or tissue sample from a subject; and (b) determining the subject's NUDT1/MTH1-status; wherein a NUDT1/MTH1-positive cell or tissue sample is indicative of an effective treatment and/or prevention of cancer. In addition, provided herein is a screening method for identifying a target of an (*S*)-enantiomer of an aminoheteroaryl compound. Furthermore, in context of this invention, the herein described compounds inhibit the biological activity of MTH1.

Drugs intended for human use require extensive toxicologic studies before they can finally be applied to patients in order to ensure that the beneficial effects outweigh potential side effects. However, certain off-target effects may only become visible after long-term use and once broader patient cohorts have been exposed to the compound. Those conditions obviously cannot be assessed completely in standard clinical trials. A chemical proteomic analysis of the BCR-Abl kinase inhibitor dasatinib for instance revealed that this compound can lead to atypical infections in chronic myelogenous leukaemia (CML) patients due to inhibition of BTK and TEC kinases.¹ Apart from potentially negative consequences for patients, certain drugs may exhibit so-called “polypharmacology”, which means that their clinical efficacy is due to simultaneous interference with several cellular proteins and respective signalling pathways which all are relevant for disease.² Thus these off-target activities can also be beneficial and may not be of concern to the patient. However, comprehensive knowledge about a drug's molecular target profile (i.e. the gene-drug interactome) may further enhance therapeutic efficacy by specific selection of patients based on defined molecular lesions and improve the prevention of side effects (“patient stratification”). Furthermore, new and distinct patient groups may selectively benefit from the off-target effect, independently of the

originally established target. Finally, knowledge about this particularly relevant off-target may also provide lead structures that enable development of more specific modulators for novel targeted therapeutics within the same or different clinical settings. If a second medical use can be discovered for a clinically established drug by such an approach both patients and pharmaceutical companies can benefit from this “drug repurposing” as the approval process with authorities can be greatly accelerated. Among target deconvolution strategies, chemical proteomics is the most powerful technique to decipher a molecule’s cellular interactome which outmatches any *in vitro* screening strategy. First and foremost, *in vitro* assays are limited to certain subproteomes such as kinases, whilst in a chemical proteomics experiment the compound of interest is exposed to a complete proteome covering all classes of enzymes including kinases, histone deacetylases but also transcription factors and many more. Notably, only chemical proteomics allows assessment of proteins in their natural abundance and state of posttranslational modification (PTM) such as phosphorylation and acetylation.

Crizotinib (PF-02341066, Xalkori®) is a novel dual inhibitor of anaplastic lymphoma kinase (ALK) and hepatocyte growth factor receptor kinase (c-Met) developed by Pfizer.³ Crizotinib is the first Food and Drug Administration (FDA) approved ALK kinase inhibitor and is used to treat patients which have been diagnosed with ALK-positive tumours. Aberrant ALK signalling induces transformation, proliferation and antagonizes cell cycle arrest and apoptosis. The molecular lesions behind oncogenic ALK activity can be associated with either ALK gene amplification, mutation or chromosomal translocations such as EML4-ALK.⁴ EML4-ALK is an oncogenic fusion protein consisting of the echinoderm microtubule-associated protein-like⁴ and ALK genes (EML4-ALK).⁵ This translocation occurs in approximately 3-13% of adenocarcinomas in non-small cell lung cancer (NSCLC) and is, apart from one reported case, considered to be mutually exclusive with mutations in EGFR or KRAS.⁶ Patients are selected for treatment with crizotinib based on a prerequisite fluorescence in situ hybridisation (FISH) analysis which detects EML4-ALK. Crizotinib also shows high clinical benefit in other ALK-positive tumours such as anaplastic large cell lymphomas (ALCL), neuroblastoma (NB) and inflammatory myofibroblastic tumours (IMT), ranging from controlled disease to significant progression-free survival and remission.⁷

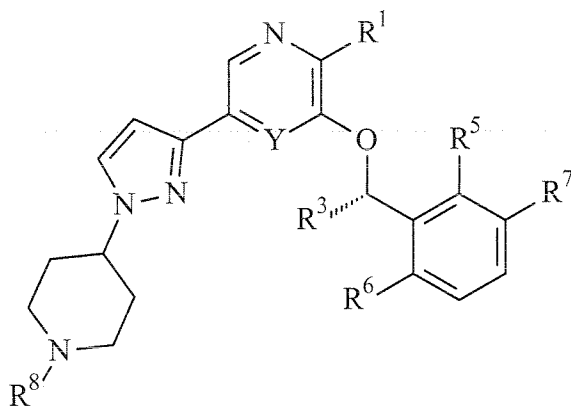
It has been shown that the (*R*)-enantiomer of crizotinib is slightly more potent than racemic crizotinib and significantly more potent than the (*S*)-enantiomer of crizotinib (herein also called “(*S*)-crizotinib”) in inhibiting c-Met activity.⁹ The different ability of (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib in inhibiting c-Met activity is consistent with specific binding as revealed in a cocrystal structure of crizotinib bound to c-Met.⁹ Indeed, clinically, only (*R*)-

crizotinib is used. In addition, as indicated above, it is the general understanding that crizotinib potentially inhibits cell proliferation in ALK-positive and c-Met-dependent cells.^{3,23}

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (World Health Organization. The Global Burden of Disease: 2004 Update. Geneva: World Health Organization; 2008; Jemal (2011) CA Cancer J Clin. 61: 69-90). In addition, novel therapies that target cancer and which are not restricted to selected cancer cells or tissue, such as those being ALK-positive and/or c-Met-dependent, but which are for example RAS-, e.g. KRAS-dependent cancers/cancer cells, are desired. Therefore, the technical problem underlying the present invention is the provision of means and methods for the medical intervention of proliferative diseases, in particular cancerous diseases/cancer.

This technical problem is solved by the embodiments provided herein and as characterized in the claims.

Accordingly, the present invention relates to an (*S*)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing cancer in a subject, wherein the compound has the following chemical structure represented by Formula (1)



Formula (1)

wherein:

R¹ is -NH₂, -NR²H, -OH or -SH;

R² is C₁₋₆ alkyl, C₂₋₆ alkenyl or C₂₋₆ alkynyl;

R³ is C₁₋₃ alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl or cyclopropyl;

Y is N or CR⁴;

R⁴ is hydrogen, halogen, C₁₋₆ alkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl or C₃₋₆ cycloalkyl;

each R⁵, R⁶ and R⁷ is independently fluorine, chlorine, bromine or iodine;

R⁸ is hydrogen or -A-B_n-X, wherein

A is a single bond, -C(=O)- or -C(=O)CH₂-;

B is C₁₋₄ alkylene, C₂₋₄ alkenylene or -(OCH₂CH₂)-;

n is 0, 1, 2, 3, 4 or 5, and

X is -NHR²; -NH₂; -SH; -OH or O-alkyl;

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

In context of the present invention, it has been surprisingly found that an (*S*)-enantiomer of an aminoheteroaryl compound (e.g. (*S*)-3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine; herein also referred to as “(*S*)-enantiomer of crizotinib”) can be used to treat or prevent RAS-dependent cancer.

It is the established scientific view that the (*S*)-enantiomer of crizotinib has no use as an anti-cancer drug since it is not a good inhibitor of the oncogenic kinase c-Met. In particular, Cui (2011) J. Med. Chem. 54: 6342-6363 discloses using crizotinib (i.e. (*R*)-crizotinib) for treating cancer. This prior art study concludes that crizotinib has antitumor efficacy in cells that express activated c-Met or ALK fusion proteins. Significantly, Cui, op. cit., further discloses that in contrast to crizotinib (i.e. (*R*)-crizotinib), the (*S*)-enantiomer of crizotinib does not, or hardly (if at all) inhibit c-Met. The finding in the prior art that crizotinib effectively inhibits c-Met (whereas the (*S*)-enantiomer does not have c-Met inhibiting activity) was attributed to the fact that specifically (*R*)-crizotinib binds to the c-Met binding pocket (Cui, op. cit.). Since inhibition of c-Met is required to treat cancer, Cui, op. cit., teaches that the (*S*)-enantiomer of crizotinib cannot treat cancer. In addition, WO 2006/021881 (A2) discloses that the pure (*R*)-enantiomer of the substance 3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine (i.e. crizotinib) inhibits c-Met considerably better than the racemate of crizotinib. Thus, the common understanding in the prior art is that an (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) does not substantially or effectively inhibit c-Met and is, thus, not suited as an anti-cancer drug. In agreement with this, in the prior art only crizotinib (i.e. (*R*)-crizotinib) and not the (*S*)-enantiomer of crizotinib has been used to inhibit growth of cancer cells.

Thus, the skilled person is taught in the prior art that these two enantiomeric compounds have disparate biological behaviours. The skilled person can therefore not draw any expectation of

activity of an (*S*)-enantiomer of an aminoheteroaryl compound (e.g. the (*S*)-enantiomer of crizotinib) from the biological activity of the (*R*)-enantiomer of crizotinib.

Further, the prior art directly and unambiguously teaches that crizotinib (*i.e.* (*R*)-crizotinib) is not suited for treating RAS (like, e.g. KRAS)-dependent cancers since activating ALK rearrangements have been found to be mutually exclusive with mutations of RAS (Gainor (2013) Clinical Cancer Research 19, 4273-4281). In agreement with this, the appended illustrative examples show that c-Met inhibition does not suppress growth of KRAS mutated SW480 cells. However, the inventors show herein, as documented in the appended illustrative examples, that an (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) is highly potent in inhibiting RAS (e.g. KRAS) mutated cancer cells *in vitro* and *in vivo*. Accordingly, the inventive finding that the (*S*)-enantiomer of crizotinib is useful in the treatment or prevention of RAS-dependent cancers could not have been predicted based on the scientific evidence available.

In other words, the prior art teaches that only the (*R*)- and not the (*S*)-enantiomer of crizotinib is useful for the treatment of cancer and that the (*R*)-enantiomer is not suited for the treatment of RAS-dependent cancers. This is in strong contrast to the finding of the present invention that an (*S*)-enantiomer of an aminoheteroaryl compound, like the pure (*S*)-enantiomer of crizotinib, is highly potent in inhibiting cancer, in particular RAS-dependent cancers.

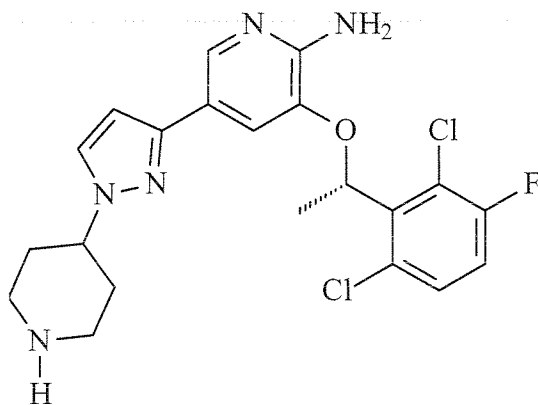
Again, as in cancer activated ALK is mutually exclusive with activated RAS, crizotinib (*i.e.* (*R*)-crizotinib) is not suited for the treatment of RAS-dependent cancers. Surprisingly, the herein provided (*S*)-enantiomer of an aminoheteroaryl compound (e.g. the (*S*)-enantiomer of crizotinib) is effective in the treatment of cancer, in particular of cancers with activating RAS mutations (e.g. activating mutations of K-RAS).

Furthermore, as is evident from the following and appended examples and figures, it was surprisingly found in context of this invention that (a) cancer cell(s) or (a) cancer tissue can successfully be treated with kinase inhibitors, even if the corresponding kinase is not active/not functional in said cell(s) and/or tissue(s). An example of such a kinase is the herein described anaplastic lymphoma kinase (ALK). It was found and documented herein that anti-ALK kinase inhibitors as disclosed herein are functional and can be used as anti-cancer agents

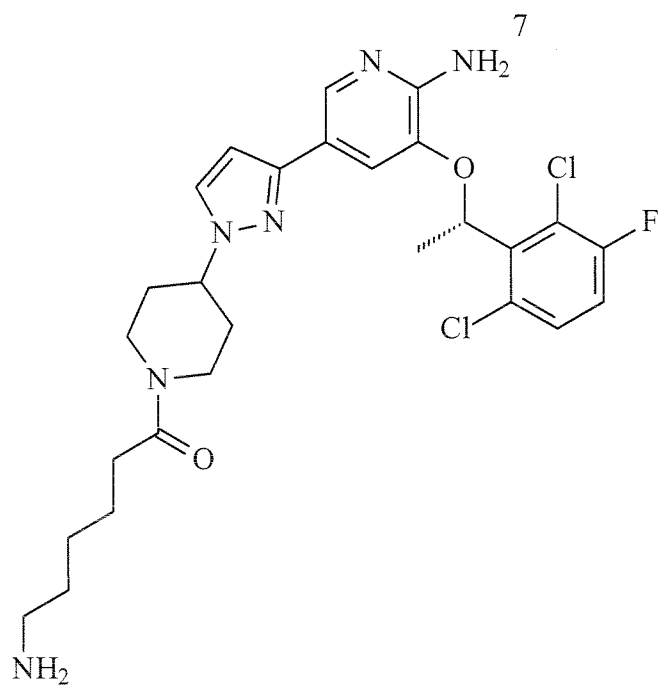
in cells wherein said kinase is not even present or is not even active or wherein said kinase has merely reduced activity. As shown and illustrated herein, it was additionally found that ALK inhibitors have a surprising target different from the anaplastic lymphoma kinase, namely the (human) mutT homologue 1/NUDT1/MTH1, i.e. a triphosphatase (here: 7,8-dihydro-8-oxoguanine-triphosphatase). This is insofar very surprising that here kinase inhibitors are successfully be used in the inhibition of the activity of an enzyme with converse catalytic activity.

In context of this invention, “NUDT1” relates to the gene and “MTH1” to the expressed protein of the above described triphosphatase.

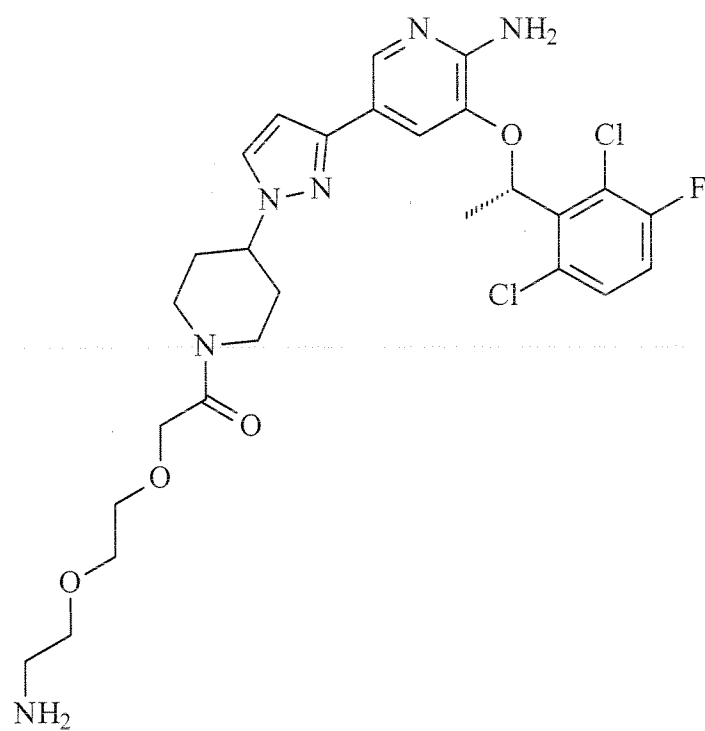
The invention relates to the above described compound of Formula (1), wherein R^1 is $-NH_2$, $-OH$ or $-SH$; R^3 is methyl; Y is CR^4 ; R^4 is hydrogen or halogen; each R^5 , R^6 and R^7 is independently fluorine or chlorine; R^8 is hydrogen or $-A-B_n-X$, wherein B is C_{1-4} alkylene or $-(OCH_2CH_2)-$; n is 1, 2 or 3, and X is $-NH_2$; or a pharmaceutically acceptable salt, solvate or prodrug thereof. In a more preferred embodiment, the invention relates to the above described compound, wherein R^8 is hydrogen; $-C(=O)CH_2CH_2CH_2CH_2CH_2-NH_2$; $-C(=O)CH_2-O-CH_2CH_2-O-CH_2CH_2-NH_2$; or $-CH_2CH_2CH_2-NH_2$; or a pharmaceutically acceptable salt, solvate or prodrug thereof. In a most preferred embodiment, the invention relates to the above described compound, wherein the compound has one of the following chemical structures represented by Formulae (2) to (5):



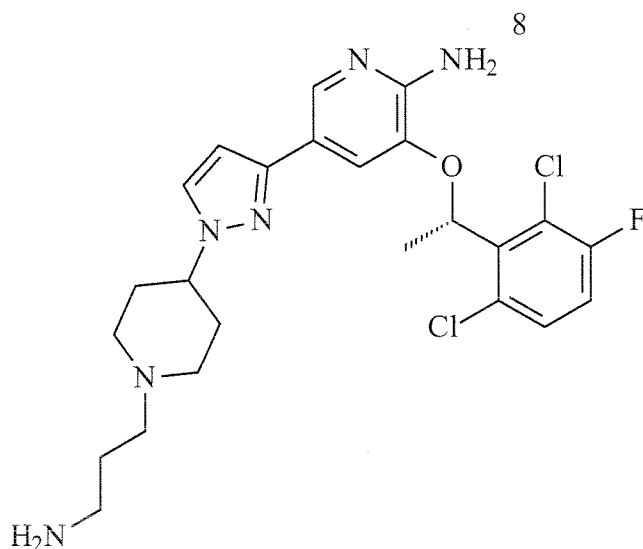
Formula (2);



Formula (3);



Formula (4) or



The invention relates to the herein defined aminoheteroaryl compound for use in treating and/or preventing cancer in a subject, wherein said aminoheteroaryl compound is in its (*S*)-enantiomer configuration.

Accordingly, the invention relates to a method of treatment and/or prevention of cancer in a subject in need of such a treatment, comprising administering to said subject a therapeutically effective amount of the herein defined (*S*)-enantiomer of an aminoheteroaryl compound. Accordingly, the present invention provides for means and methods for the treatment of subjects in need of such a treatment (either curative or preventive) which suffer from cancer. It is noted that the treatment and/or prevention is independent of the ALK-status and/or the c-Met-status of the cancer. Accordingly, the cancer to be treated and/or prevented may be an ALK-negative cancer (i.e. a cancer which does not have an activating ALK aberration) and/or a c-Met-negative cancer.

Thus, one aspect of the invention relates to the above described (*S*)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing cancer in a subject, wherein the treatment and/or prevention is independent of the ALK-status and/or the c-Met-status of the cancer cell or tissue of said subject. The ALK-status may be the level of ALK biological activity and/or the level of ALK expression. The level of ALK biological activity may be determined, e.g. by measuring the ALK kinase activity. To measure the ALK kinase activity in a patient, cancer cells or tissues of said patient may be isolated and the phosphorylation of downstream targets of ALK may be determined by Western blot using phosphospecific antibodies. The level of ALK expression may be determined, e.g., by polymerase chain reaction (PCR), real-time PCR (RT-PCT) or Western blot. The ALK-status of a cancer cell or

tissue is positive, if the ALK biological activity and/or the ALK expression is increased in said cancer cell or tissue as compared to a sample of a healthy control person. It is commonly known in the art that the ALK-status (*i.e.* the level of ALK biological activity and/or the level of ALK expression) depends on the existence of an activating aberration (e.g. a chromosomal translocation) within the ALK gene. For example, an ALK-positive cancer cell or tissue relates to a cancer cell or tissue, wherein the ALK gene has an activating aberration (such as EML4-ALK or kinase domain activating mutations, e.g. ALK F1174L). In line with this, an ALK-negative cancer cell or tissue relates to a cancer cell or tissue wherein the ALK gene has not an activating aberration (such as EML4-ALK or kinase domain activating mutation). The existence of an activating ALK aberration may be determined, e.g., by sequencing, fluorescence-in-situ hybridization (FISH), by polymerase chain reaction (PCR), real-time PCR (RT-PCT) or Western blot.

As described above, the herein provided (*S*)-enantiomer of an aminoheteroaryl compound is useful in the treatment of cancer independent of the cancer's c-Met status. In accordance with the present invention, the c-Met-status may be the level of c-Met biological activity and/or the level of c-Met expression. The level of c-Met biological activity may be determined by measuring the kinase activity of c-Met. To measure the c-Met kinase activity in a patient, cancer cells or tissues of said patient may be isolated and the phosphorylation of downstream targets of c-Met may be determined by Western blot using phosphospecific antibodies. The level of c-Met expression may be determined, e.g., by PCR, RT-PCT or Western blot. The c-Met-status of a cancer cell or tissue is positive, if the c-Met biological activity and/or the c-Met expression is increased in said cancer cell or tissue as compared to a sample of a healthy control person. For example, the c-Met status may be positive as a result of activating mutations in c-Met. Such activating mutations may result in an increased biological activity of c-Met in a cancer cell or tissue as compared to the biological activity of c-Met in a cell or tissue of a healthy control subject.

Surprisingly, an (*S*)-enantiomer of an aminoheteroaryl compound (like the (*S*)-enantiomer of crizotinib) as provided herein is suitable for the treatment of a cancer independent of the ALK- or c-Met-status of the cancer (*i.e.* independent of the level of the biological activity or the level of the expression of ALK or c-Met in the cancer cell or tissue) as documented in the appended examples in *in vitro* and *in vivo* experiments. Thus, said cancer cell or tissue may be ALK-negative (*i.e.* may not have an activating ALK aberration) and/or c-Met-negative (*i.e.*

may not have an increased expression of c-Met or kinase activating mutations as compared to a cell or tissue of a healthy control subject). This is surprising because crizotinib (i.e. (*R*)-crizotinib) is only described to be effective in ALK positive and c-Met positive cancer cells.

As described herein and illustrated in the appended examples, it was surprisingly found that the dual inhibitor of anaplastic lymphoma kinase (ALK) and hepatocyte growth factor receptor kinase (c-Met), crizotinib, is a highly potent inhibitor of the human MTH1 homologue 1 (MTH1) 7,8-dihydro-8-oxoguanine-triphosphatase which has been linked to oncogene-induced malignant transformation and cancer cell escape from senescence. Furthermore, it has astonishingly been found that the (*S*)-enantiomer of crizotinib is a particularly good MTH1 inhibitor, since this enantiomer showed considerably better MTH1 inhibitory activity than racemic or (*R*)-crizotinib.

These findings are highly surprising, since, at present, it is the general understanding that crizotinib effectively inhibits cell proliferation only in ALK-positive and c-Met-dependent cells.^{3,23} Furthermore, considering that clinically, only (*R*)-crizotinib is used as this enantiomer has been shown to be considerably more potent than the (*S*)-enantiomer in inhibiting c-Met kinase activity,⁹ the herein described invention is even more surprising.

Comprehensive knowledge about a drug's cellular target profile is a key prerequisite for patient stratification, thereby maximising treatment efficacy whilst minimizing side effects. On top of that, drugs which have proven to be particularly active in the treatment of devastating diseases such as cancer may still have an additional or even completely unknown mode of action which if revealed could lead to the development of novel therapeutics.

Crizotinib abrogates malignant ALK signalling by binding to the adenosine triphosphate (ATP) binding pocket within the kinase active site and therefore acts as an (ATP) competitive kinase inhibitor. The inventors of the present invention hypothesised that crizotinib's high efficacy could at least partially be due to interference with several targets other than ALK which are relevant for cancer cell survival and set out to profile crizotinib by chemical proteomics. In particular, as shown in the appended examples, to investigate potentially relevant on- or off-targets of the clinically highly efficient and first-in-class dual ALK/c-Met kinase inhibitor crizotinib (Xalkori®), the inventors of the present invention applied a chemical proteomics approach interrogating several tumour cell lines with different genetic lesions. Investigating several human tumour cell lines the inventors of the present invention have surprisingly identified several unknown off-targets of crizotinib including the

unexpected non-kinase target human MTH1 which has been linked to malignant transformation induced by mutant RAS and which has been shown to enable tumours to overcome the oncogene-induced senescence (OIS) barrier.

In particular, as demonstrated in the appended illustrative examples, inhibition of MTH1 catalytic activity by racemic crizotinib could be confirmed in an *in vitro* luminescence-based enzymatic assay indicating low nanomolar potency. Racemic crizotinib also induced DNA damage in MRC-5 lung fibroblasts as demonstrated by the comet assay. In addition, (*S*)-crizotinib but not (*R*)-crizotinib yielded a significant tail moment in the comet assay. At the same time, staining for 53BP1, a specific marker for DNA damage, was increased when cells were treated with the (*S*)-enantiomer of crizotinib, which was in line with the effect observed for anti-MTH1 siRNA. Considering the strong link between MTH1 and the expression of oncogenic RAS, reactive oxygen species (ROS), oxidative damage and tumour development, the data provided herein surprisingly suggests that small-molecule MTH1 inhibitors such as crizotinib, preferably racemic crizotinib, or more preferably the (*S*)-enantiomer of crizotinib, could also be applied to the treatment of several diverse ALK-negative cancers.

It is noted that the term “crizotinib” is also known as “PF-2341066” and relates to the (*R*)-enantiomer of the chemical substance 3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine (see, e.g., Zou (2007) Cancer Res. 67: 4408-4417³; Cui (2011) J. Med. Chem. 54: 6342-6363⁹; and Christensen (2007) Mol Cancer Ther. 6: 3314-3322²³). The terms “crizotinib”, “(*R*)-crizotinib”, and “(*R*)-enantiomer of crizotinib” are used interchangeably herein. To simplify matters, as used herein, the terms “racemic crizotinib” or “racemate of crizotinib” relate to the racemic form of the chemical substance 3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine. In line with this, as used herein, the terms “(*S*)-crizotinib” and “(*S*)-enantiomer of crizotinib” relate to the (*S*)-enantiomer of the chemical substance 3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine.

As demonstrated in the illustrative appended examples, the potency to inhibit MTH1 catalytic activity of racemic crizotinib, (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib is considerably different. In particular, in an assay to determine inhibition of MTH1 catalytic activity, the IC₅₀ value determined for racemic crizotinib was about 10 times higher than the one observed for the enantiomerically pure (*R*)-crizotinib. Furthermore, the (*S*)-enantiomer of crizotinib indicated more than 100-fold higher inhibitory potency than (*R*)-crizotinib.

Apparently, the (*S*)-conformation leads to an increased affinity of an aminoheteroaryl compound to MTH1. Accordingly, in context of the present invention it has been surprisingly demonstrated that crizotinib, more preferably racemic crizotinib, and even more preferably the pure (*S*)-enantiomer of crizotinib are highly potent inhibitors of MTH1, an enzyme which has been linked to the development, progression and maintenance of RAS-driven cancer.

Therefore, the present invention provides for the surprising finding, that the (*S*)-enantiomer of an aminoheteroaryl compound of the invention (such as the (*S*)-enantiomer of crizotinib) effectively inhibits the biological activity (in particular the catalytic activity) of MTH1. Considering that MTH1 has been linked to malignant transformation induced by mutant RAS, it is one aspect of the present invention that the (*S*)-enantiomer of an aminoheteroaryl compound of the invention (such as the (*S*)-enantiomer of crizotinib) can be used in treating and/or preventing cancer. In addition, since inhibition of MTH1 is independent of the cancer's ALK- and/or c-Met-status, it is a further aspect of the present invention that the (*S*)-enantiomer of an aminoheteroaryl compound of the invention (such as the (*S*)-enantiomer of crizotinib) can be used in treating and/or preventing ALK-negative and/or c-Met-negative cancer.

As described herein, the invention relates to an (*S*)-enantiomer of an aminoheteroaryl compound or Formula (1) (such as the (*S*)-enantiomer of crizotinib) or a pharmaceutically acceptable salt, solvate or prodrug thereof, for use in treating and/or preventing cancer. According to the present invention, the treatment and/or prevention is independent of the ALK-status and/or the c-Met-status of the cancer to be treated.

One embodiment of the invention relates to the compound for the use of the invention, wherein

R^1 is $-NH_2$, $-OH$ or SH ;

R^3 is methyl;

Y is CR^4 ;

R^4 is hydrogen or halogen;

each R^5 , R^6 and R^7 is independently fluorine or chlorine;

R^8 is hydrogen or $-A-B_n-X$, wherein

B is C_{1-4} alkylene or $-(OCH_2CH_2)-$;

n is 1, 2 or 3, and

X is $-NH_2$;

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

A further embodiment of the invention relates to the compound for the use of the invention, wherein

R^8 is hydrogen;

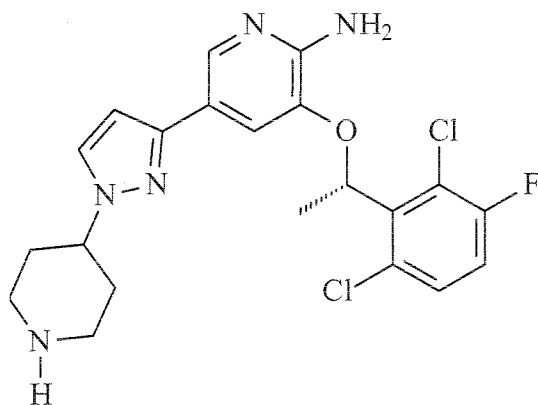
$-C(=O)CH_2CH_2CH_2CH_2CH_2-NH_2$;

$-C(=O)CH_2-O-CH_2CH_2-O-CH_2CH_2-NH_2$; or

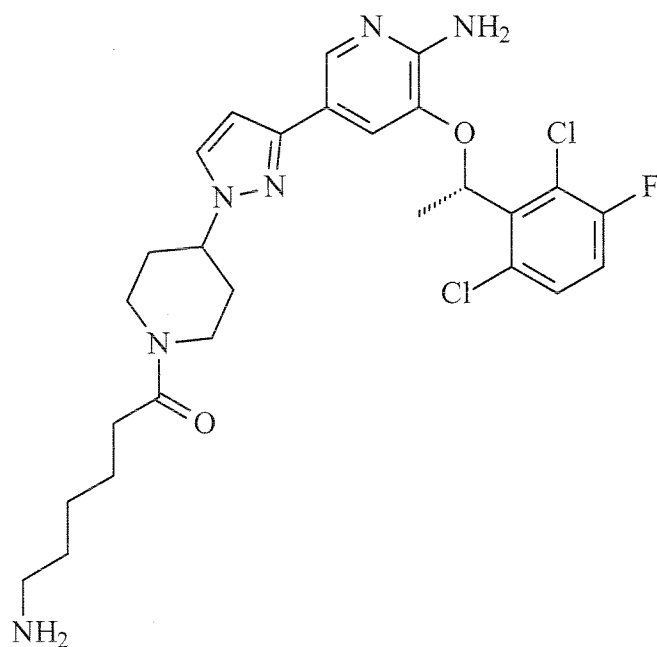
$-CH_2CH_2CH_2-NH_2$;

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

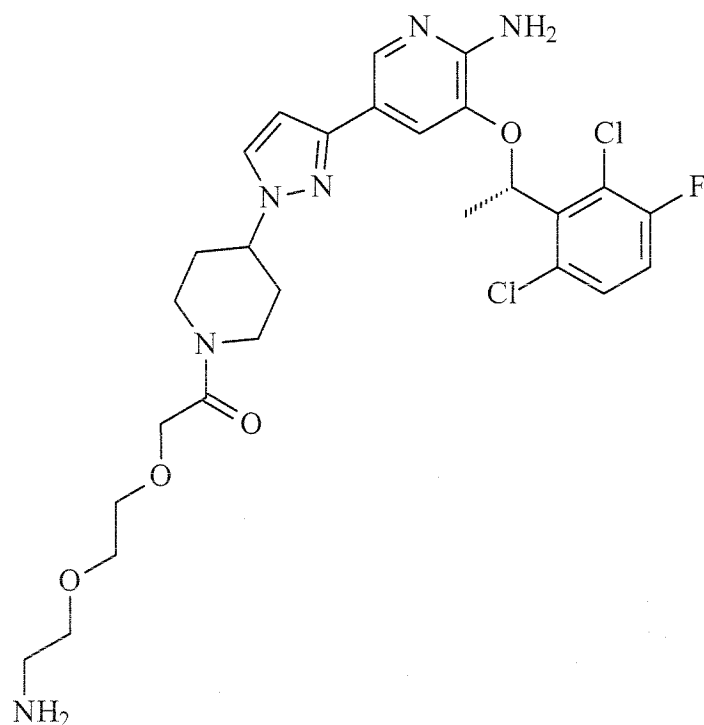
Another embodiment of the invention relates to the compound for the use of the invention, wherein the compound has one of the following chemical structures represented by Formulae (2) to (5):



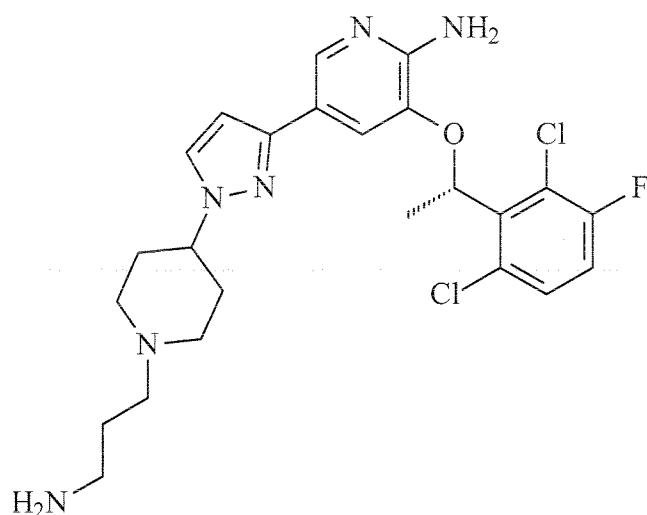
Formula (2);



Formula (3);



Formula (4) or



Formula (5).

It is also envisaged that the (*S*)-enantiomer of an aminoheteroaryl compound of the invention may be administered to a subject as compounds per se in their use as pharmacophores or pharmaceutical compositions or may be formulated as medicaments.

Accordingly, the present invention further relates to a pharmaceutical composition comprising the compound for the use of the invention and a pharmaceutically acceptable excipient. Thus, one aspect of the invention relates to a pharmaceutical composition for use in treating and/or preventing cancer, wherein said pharmaceutical composition comprises the (*S*)-enantiomer of

an aminoheteroaryl compound as provided herein and a pharmaceutically acceptable excipient.

One aspect of the invention relates to a pharmaceutical composition comprising the (*S*)-enantiomer of an aminoheteroaryl compound provided herein and a pharmaceutically acceptable excipient, for use in treating and/or preventing cancer in a subject, wherein the treatment and/or prevention is independent of the ALK-status and/or the c-Met-status of the cancer cell or tissue of said subject. Said ALK-status may be the level of ALK biological activity and/or the level of ALK expression. In line with this, said c-Met-status may be the level of c-Met biological activity and/or the level of c-Met expression. Methods for determining the level of the biological activity or the level of the expression of ALK or c-Met are described herein above and below.

Thus said cancer to be treated may be ALK-negative (i.e. may not have an activating ALK aberration) and/or c-Met-negative. In accordance with the present invention, said pharmaceutically acceptable excipient may be a carrier, diluent, filler, desintegrant, lubricating agent, binder, colorant, pigment, stabilizer, preservative or antioxidant.

As described herein, one embodiment of the invention relates to the pharmaceutical composition of the invention, comprising at least two components each having one of the chemical structures represented by Formulae (1) to (5). Another embodiment of the invention relates to the pharmaceutical composition of the invention, comprising at least two components each having one of the chemical structures represented by Formulae (2) to (5). As indicated above, the invention relates to an (*S*)-enantiomer of an aminoheteroaryl compound and a pharmaceutical composition for use in treating and/or preventing cancer in a subject. The invention further relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein the treatment and/or prevention of cancer in a subject is independent of the ALK-status and/or the c-Met-status of the cancer cell or tissue of said subject. Thus, the cancer to be treated and/or prevented may be ALK-negative (i.e. may not have an activating ALK aberration) and/or may be c-Met-negative.

Crizotinib is a dual inhibitor of anaplastic lymphoma kinase (ALK) and hepatocyte growth factor receptor kinase (c-Met).³ At present, crizotinib is exclusively used to treat patients who have been diagnosed with ALK-positive tumours. The molecular lesions behind oncogenic ALK activity can be associated with either ALK gene amplification, mutation or chromosomal translocations such as EML4-ALK.⁴ EML4-ALK is an oncogenic fusion

protein consisting of the echinoderm microtubule-associated protein-like 4 and ALK genes (EML4-ALK).⁵

As indicated above, in context of the present invention it has been surprisingly found that aminoheteroaryl compounds (such as crizotinib), and preferably the (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) are able to inhibit the biological activity of MTH1 and can therefore also be used to treat and/or prevent cancer in a subject independent of the ALK-status and/or c-Met-status of the cancer. Accordingly, a further embodiment of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said cancer cell or tissue of said subject does not have a gene mutation and/or a chromosomal translocation of ALK. One aspect of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said chromosomal translocation is EML4-ALK.

This translocation occurs in approximately 3-13% of adenocarcinomas in non-small cell lung cancer (NSCLC) and is, apart from one reported case, considered to be mutually exclusive with mutations in EGFR or KRAS.⁶ Furthermore, as ALK rearrangements have been found to be mutually exclusive with mutations in RAS or EGFR family genes in lung cancer patients (Gainor (2013) Clinical Cancer Research 19, 4273-4281), the ALK kinase inhibitor (*R*)-crizotinib is not suited for the treatment of patients bearing mutations in KRAS or EGFR. It is noteworthy that even patients that have developed resistance toward (*R*)-crizotinib [Xalkori®] do not seem to acquire mutations in KRAS or EGFR (Gainor, op., cit.), which further underscores the lack of rationale to treat patients whose cancer has RAS mutations or increased EGFR expression with (*R*)-crizotinib [Xalkori®]. In agreement with this, the appended illustrative examples show that c-Met inhibition does not suppress growth of KRAS mutated SW480 cells. In contrast, the (*S*)-enantiomer of crizotinib decreases cell growth of SW480 cells *in vitro* as well as in an *in vivo* mouse model. These results demonstrate that in contrast to crizotinib (i.e. (*R*)-crizotinib) the (*S*)-enantiomer of crizotinib is useful for the treatment of RAS positive cancers.

It has been surprisingly demonstrated in the appended examples that the pure (*S*)-enantiomer of crizotinib is a highly potent inhibitor of MTH1, an enzyme which has been linked in the prior art to the development, progression and maintenance of RAS-driven cancer.¹³ It is mentioned that the prior art recently demonstrated that MTH1 might also be a promising

target for adenocarcinomas expressing EGFR, as the micro-RNA MiR-145 which suppresses both EGFR and MTH1 is downregulated in these tumours.³⁷ As also shown in the prior art, reexpression of MiR-145 led to a downregulation of EGFR and MTH1 on both mRNA and protein level and impaired the growth of EGFR-positive cell lines. In addition, as shown in the appended examples, the (*S*)-enantiomer of crizotinib efficiently inhibited colony formation of human colon adenocarcinoma cells (SW480) as well as of pancreatic cancer cells (PANC1), both having an activating RAS mutation. Consistent with these results, stable knockdown of MTH1 significantly reduced colony formation of SW480 cells. Furthermore, as described herein, a mouse xenograft study using human colon adenocarcinoma cells (SW480) demonstrates that the (*S*)-enantiomer of crizotinib is able to impair tumour progression. In particular, administering of the (*S*)-enantiomer of crizotinib resulted in a reduction in tumour volume of more than 50%. As also evident from this experiment, the (*S*)-enantiomer of crizotinib is well-tolerated as animals behaved normally and no significant change in haematological parameters or body weight was observed.

Thus, one embodiment of the present invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein the cancer cell or tissue of said subject has an activating RAS mutation and/or an activating EGFR mutation. One particular embodiment of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein the cancer cell or tissue of said subject has an activating RAS mutation. Said activating RAS mutation may be an activating KRAS mutation (e.g. G12D, G12V, or G12C). Treating cells transformed with mutant RAS by targeting MTH1 is in line with the prior art showing that transformation of cells by mutant RAS can lead to increased production of ROS.¹² As for normal cells, oxidative damage caused by ROS can force cancer cells into a state of quiescence or senescence (OIS), and eventually apoptosis. To overcome senescence, RAS-transformed cells upregulate MTH1 which protects the cells from oxidative DNA damage. For instance, it has been described in the prior art that human skin fibroblasts transfected with HRAS undergo senescence, but this phenotype can be rescued by concomitant overexpression of MTH1.¹² Consequently, MTH1 suppression causes proliferative defects in cancer cells expressing mutant RAS.¹³ As MTH1^{-/-} knockout mice show a very mild phenotype,¹⁴ targeting MTH1 with small molecules may provide a novel and well-tolerated therapeutic option for the difficult to treat RAS mutant cancers. Indeed, as also shown in the appended examples, clinically used (*R*)-enantiomer of crizotinib exhibited higher toxicity than the (*S*)-enantiomer

of crizotinib on non-transformed cells. In agreement with this and as also shown in the appended illustrative examples, the (*S*)-enantiomer of crizotinib is less toxic to wildtype cells or cells which are only immortalized by telomerase as compared to transformed cells. This result underscores the cancer-specific effect of the (*S*)-enantiomer of crizotinib.

Activated RAS GTPase signalling is a critical driver of oncogenic transformation and malignant disease (Pylayeva-Gupta (2011) Nat Rev Cancer 11, 761-774). About 20-30% of human cancers contain mutations in one RAS isoform, (Parada (1982) Nature 297, 474-478; Der (1982) Proceedings of the National Academy of Sciences 79, 3637-3640) which is accompanied by poor prognosis and low overall survival, highlighting the urgent need to identify new inhibitors. However, the structure of the RAS proteins makes them poor targets for small molecules. As an alternative, cellular models of RAS-dependent cancers have been used in the prior art to develop specific inhibitors such as SCH51344, but the molecular targets remain mostly enigmatic (Kumar (1995) Cancer Research 55, 5106-5117). Direct modulation of RAS activity by small molecules has posed a significant challenge in drug discovery. Therefore, alternative approaches have been used in the prior art, for example to interfere with RAS processing, which is regulated via posttranslational modifications, e.g. palmitoylation, to prevent maturation and translocation of the active protein to the plasma membrane (Xu (2012) Blood 119, 1032-1035; Dekker (2010) Nat Chem Biol 6, 449-456). In addition to these targeted approaches, phenotypic screens have been employed in the prior art to search for small molecules that selectively target RAS-transformed cancer cells but do not affect the growth of untransformed cells (Yagoda (2007) Nature 447, 865-869). The benefit of such a strategy is that active compounds are already being selected on the basis of their specificity for cancer cells and their cellular activity. In 1995, this approach led to the discovery of a pyrazoloquinoline compound termed SCH51344 that suppressed the anchorage-independent growth of RAS-transformed fibroblasts as well as human colon carcinoma and pancreatic cancer cell lines (Kumar (1995) Cancer Research 55, 5106-5117). Significantly, as documented in the appended examples, the herein provided (*S*)-enantiomer of an aminoheteroaryl compound (e.g. the (*S*)-enantiomer of crizotinib) is considerably more effective in the treatment of RAS-dependent cancer cells than the compound SCH51344.

Several proteins play a role in the repair of damaged DNA. These proteins include DNA ligase, PCNA (a sliding clamp), as well as p53, p21, and MLH1. Studies in model systems have shown that the antiproliferative effect of MTH1 suppression may depend on p53, a well-

known tumour suppressor gene (Rai (2009) Proceedings of the National Academy of Sciences 106, 169-174; Rai (2010) Mutation Research/Genetic Toxicology and Environmental Mutagenesis 703, 71-81). In particular, loss of p53 function was demonstrated to prevent senescence induction by MTH1 genetic silencing. Therefore, it is likely that patients with functional p53 signalling may benefit preferably from MTH1 inhibitor treatment. However, the appended illustrative examples indicate that the p53 status as well as the presence or absence of functional MLH1 do not affect the activity of the (*S*)-enantiomer of crizotinib. However, loss of p21 increases the sensitivity of cancer cells (e.g. HCT116 cells) toward treatment with the (*S*)-enantiomer of crizotinib. Accordingly, it is likely that patients with non-functional p21 signalling may benefit preferably from treatment with the (*S*)-enantiomer of an aminoheteroaryl compound of the present invention. Thus, one aspect of the invention is directed to the herein provided (*S*)-enantiomer of an aminoheteroaryl compound or the herein described pharmaceutical composition for use in treating and/or preventing cancer in a subject, wherein in the cancer cell or tissue of said subject the biological activity and/or expression of p21 is reduced or absent. In this regard, the term “reduced” means that the biological activity of p21 and/or the expression of p21 is reduced as compared to the biological activity or expression of p21 in a cell or tissue sample of a healthy control subject (e.g. of a healthy control person). Human p21 inhibits cyclin-CDK2 and cyclin-CDK4 complexes, regulating cell cycle progression in G1 phase. Reduced p21 expression has been implicated in a variety of human cancers including those of the prostate, bladder, and esophagus. The tumor suppressor p21 mediates its various biological activities primarily by binding to and inhibiting the kinase activity of the cyclin-dependent kinases (CDKs) CDK2 and CDK1 (also known as CDC2). Thus, in order to determine the biological activity of p21, the ability of purified p21 to inhibit kinase activity of CDK2 or CDK1 may be determined. CDK activity (e.g. activity of CDK2) may be monitored by determining substrate phosphorylation using radioactively-labeled ATP. For example, for measurement of Cdk2 activity, the [33P]ATP Scintillation Proximity Assay of PerkinElmer may be used (see, e.g., <http://www.perkinelmer.com/pages/020/proximitynews/enzymes/measurementofcdk2.xhtml>). The expression of p21 may be determined, e.g., by PCR, RT-PCT or Western blot.

In one embodiment of the invention, the cancer cell or tissue of the subject to be treated with the herein provided (*S*)-enantiomer of an aminoheteroaryl compound or pharmaceutical composition has the genetic constitution p21^{+/-} (i.e. one allele of p21 is deleted or inactivated).

In another embodiment of the invention, said cancer cell or tissue has the genetic constitution $p21^{-/-}$ (i.e. both alleles of p21 are deleted or inactivated).

As described above, the present invention relates to an (*S*)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing cancer in a subject. One aspect of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said subject is a mammal. A further aspect of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said mammal is a human patient.

As described herein and illustrated in the appended examples, it has been found that crizotinib, preferably racemic crizotinib, and more preferably the (*S*)-enantiomer of crizotinib as well as its structural derivatives could be applied in the treatment of a variety of distinct tumour types apart from ALK/Met-driven lung cancer but also RAS-driven colon cancer, breast cancer, lung cancer, pancreatic cancer, Ewing's sarcoma and many more. As shown herein, the (*S*)-enantiomer of crizotinib efficiently inhibits growth and progression of RAS-dependent cancer cells. Therefore, it is prioritized to use the (*S*)-enantiomer of an aminoheteroaryl compound of the present invention to treat and/or prevent cancer having an activating RAS mutation. This cancer may be, e.g., colon cancer. Thus, one embodiment of the present invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein the cancer is selected from the group consisting of colon cancer, lung cancer, breast cancer, leukaemia, lymphoma, skin cancer, liver cancer, pancreatic cancer, brain cancer, kidney cancer, neuroblastoma, Ewing's sarcoma, prostate cancer, bladder cancer and esophagus cancer.

It has been shown in the prior art that triple negative breast cancer cell lines are sensitive to oxidative DNA damage and that this damage can sensitize these cancers to other chemotherapeutics such as PARP inhibitors.¹⁸ Therefore, MTH1 inhibitors which are thought to induce oxidative DNA lesions could be used to treat these tumour types. In addition, it has been reported that the BRCA1 and BRCA2 genes which are mutated in some types of breast cancer are involved in the repair of oxidative DNA damage including 8-oxo-guanine-based lesions.^{17,39} Consequently, one aspect of the invention relates to use the herein identified MTH1 inhibitors to target these tumours. In this context, the MTH1 inhibitors could also synergize with other standard-of-care agents such as PARP inhibitors. Thus, it is envisaged in

context of the present invention to use MTH1 inhibitors for treating and/or preventing breast cancer, preferably triple-negative breast cancer. Accordingly, the present invention further relates to the compound for the use of the invention, or the pharmaceutical composition of the invention for treating and/or preventing breast cancer in a subject. One embodiment of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said breast cancer lacks expression of estrogen receptor- α and progesterone receptor and lacks overexpression or amplification of the HER2/NEU oncogene (i. e, triple-negative breast cancer). A particular aspect of the present invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said breast cancer has a BRCA1 and/or BRCA2 gene mutation. The breast cancer or the lung cancer which is to be treated and/or prevented with the compound for the use of the invention or the pharmaceutical composition of the invention may further be EGFR-dependent.

Based on the physiological effects of MTH1 suppression, MTH1 inhibitors are likely to synergise with radiotherapy and/or chemotherapy, preferably with DNA damaging compounds, compounds which interfere with DNA repair mechanisms, or compounds which induce the production or inhibit the clearance of ROS.

Thus, one embodiment of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said compound or pharmaceutical composition is co-administered with radiation and/or chemotherapy. One aspect of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said chemotherapy is a DNA damaging drug, an alkylating agent, a DNA intercalator, a topoisomerase inhibitor, an agent which confers oxidative damage to DNA, a cytotoxic compound, an antimetabolite, a compound which interferes with DNA repair mechanisms, an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 kinase inhibitor, a PARP inhibitor, an EGFR inhibitor, a DNA-dependent protein kinase inhibitor, a generic base excision repair inhibitor, a DNA polymerase beta inhibitor, a O-6-methylguanine methyltransferase (MGMT) inhibitor, a survivin suppressant, a compound generating reactive oxygen species (ROS), an antimitotic compound, or a combination of any of the foregoing. As indicated above, the compound for the use of the invention, or the pharmaceutical composition of the invention may be co-administered with chemotherapy. A preferred aspect of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said chemotherapy is

a PARP inhibitor and/or an EGFR inhibitor. However, beside PARP inhibitors and/or EGFR inhibitors, several other chemotherapeutic drugs may be co-administered with the compound of the invention or the pharmaceutical composition of the invention. Thus, another aspect of the invention relates to the compound for the use of the invention, or the pharmaceutical composition of the invention, wherein said chemotherapy is cyclophosphamide, temozolomide, melphalan, carmustine, busulfan, cisplatin, procarbazine, anthracyclines, camptothecin, irinotecan, etoposide, hydrogen peroxide, resorcinol, quinones, methotrexate, 5-fluorouracil, thalidomide, lenalidomide, pomalidomide, olaparib, ABT-888, neocarzinostatin, bleomycin, decitabine, 5-azacytosine, methoxyamine hydrochloride (TRC102), lomeguatrib, piperlongumine, quercetin, vincristin, taxol, mitoxantrone, YM155, erlotinib, gefitinib, lapatinib, or a combination of any of the foregoing. Accordingly, as described herein, the herein defined (*S*)-enantiomer of an aminoheteroaryl compound can be co-administered with several chemotherapeutic drugs including DNA damaging drugs such as alkylating agents (e.g. cyclophosphamide, temozolomide, melphalan, carmustine, busulfan, cisplatin, procarbazine and others), DNA intercalators (e.g. anthracyclines) or topoisomerase inhibitors (e.g. camptothecin, irinotecan, etoposide), agents which confer oxidative damage to DNA (e.g. hydrogen peroxide, resorcinol, quinones) other cytotoxic compounds (e.g. antimetabolites including methotrexate, 5-fluorouracil, thalidomide and analogues thereof, neocarzinostatin, bleomycin, decitabine, 5-azacytosine), compounds which interfere with DNA repair mechanisms (e.g. ATM-, ATR- or CHK1/2 kinase inhibitors, PARP inhibitors, DNA-dependent protein kinase inhibitors, generic base excision repair inhibitors such as methoxyamine hydrochloride (TRC102), DNA polymerase beta inhibitors, O-6-methylguanine methyltransferase (MGMT) inhibitors (e.g. lomeguatrib), compounds generating reactive oxygen species (ROS) (e.g. piperlongumine, quercetin), antimitotic compounds such as vincristin or taxol, EGFR inhibitors (e.g. erlotinib, gefitinib, lapatinib) as well as mitoxantrone and YM155.

As provided herein, crizotinib, more preferably racemic crizotinib, and even more preferably the pure (*S*)-enantiomer of crizotinib are highly potent inhibitors of MTH1. This is of particular interest as a) small molecule inhibitors of MTH1 have not been reported so far, b) patients who are to be treated with crizotinib are selected by their c-Met-, or more preferably, ALK-expression status whilst not considering MTH1, and c) as demonstrated in the appended examples, introduction of aminoalkyl substituents at the piperidine nitrogen of crizotinib is well tolerated with respect to both ALK and MTH1 inhibition. The latter finding also suggests

that compounds disclosed in WO 2008/053157, WO 2006/021881, WO 2006/021886 and WO 2006/021884 are potent and bioavailable MTH1 inhibitors. Accordingly, one aspect of the invention relates to the use of the compounds disclosed in WO 2008/053157, WO 2006/021881, WO 2006/021886 and WO 2006/021884 for inhibiting MHT1 and thus, treating and/or preventing cancer.

As described herein, it has been found that the (*S*)-enantiomer of an aminoheteroaryl compound effectively inhibits the biological activity of MTH1. Therefore, the compound of the invention can be used to treat and/or prevent cancers, in particular cancers wherein MTH1 is involved in the development of the cancer and/or progression of the cancer. To determine whether a MTH1 is involved in the development and/or progression of a cancer, one can analyze the status (e.g. the genetic constitution, expression and/or activity) of MTH1. The MTH1-status of a cancer represents an appropriate indicator for the involvement of MTH1 in the development and/or progression of said cancer. Accordingly, it is a further aspect of the invention to stratify cancer patients with respect to their MTH1-status.

Thus, a further embodiment of the invention relates to an *in vitro* method for determining the effectiveness of the compound for the use of the invention, or the pharmaceutical composition of the invention, the method comprising the steps of:

- (a) obtaining a cell or tissue sample from a subject; and
- (b) determining the subject's NUDT1/MTH1-status;

wherein a NUDT1/MTH1-positive cell or tissue sample is indicative of an effective treatment and/or prevention of cancer.

In one aspect of this *in vitro* method, said NUDT/MTH1-status is the level of MTH1 biological activity and/or the level of MTH1 expression. The level of MTH1 biological activity may be monitored by purifying MTH1 from said cell or tissue sample from said subject and measuring the production of PPi generated by MTH1-mediated 8-oxo-dGTP hydrolysis. In particular, purified MTH1 may be contacted with 8-oxo-dGTP and the production of PPi may be measured using the PPiLight Inorganic Pyrophosphate Assay (Lonza Rockland Inc.). In order to determine whether the herein described (*S*)-enantiomer of crizotinib or the herein described pharmaceutical composition is suitable for the treatment of a cancer patient, the level of MTH1 biological activity (*i.e.* the quantity of produced PPi) of MTH1 within a sample of said cancer patient may be compared to the level of MTH1 biological activity (*i.e.* the quantity of produced PPi) of MTH1 within a sample of a healthy

control person. The cell or tissue sample of the cancer patient is “NUDT1/MTH1-positive” if the level of MTH1 biological activity (*i.e.* the amount of produced PPI) is higher in the sample of the cancer patient as compared to the sample of the healthy control person. In this case the herein described (*S*)-enantiomer of an aminoheteroaryl compound or the herein described pharmaceutical composition is suitable for the treatment of said cancer patient.

The level of MTH1 expression may be determined, for example, by PCR, RT-PCT or western blot. The cell or tissue sample is “NUDT1/MTH1-positive” if the expression of MTH1 (*i.e.* the amount of the MTH1 mRNA or of the MTH1 protein) is higher in a sample of a cancer patient as compared to a sample of a healthy control person. In this case the herein described (*S*)-enantiomer of an aminoheteroaryl compound or the herein described pharmaceutical composition is suitable for the treatment of said cancer patient. In addition, as described in more detail below, for the determination of the status of MTH1, several other detection methods can be applied.

One aspect of the invention relates to the *in vitro* method of the invention, further comprising the step of:

(c) determining the subject's RAS-status;

wherein a cell or tissue sample positive for NUDT1/MTH1 and positive for an activating RAS mutation are indicative of an effective treatment and/or prevention of cancer. Accordingly, in this context, the term “RAS-status” relates to the existences of an activating RAS mutation. Accordingly, one aspect of the invention relates to the above described *in vitro* method, further comprising the step of:

(c) determining whether said cell or tissue sample has an activating RAS mutation;

wherein a cell or tissue sample positive for NUDT1/MTH1 and positive for an activating RAS mutation are indicative of an effective treatment and/or prevention of cancer.

An activating RAS mutation is an activating aberration of RAS. In particular, an activating RAS mutation relates to a mutation within RAS which leads to an increased biological activity (*i.e.* catalytic activity) of RAS. The person skilled in the art readily knows several activating RAS mutations. Examples for activating RAS mutations are, *e.g.*, the G12 mutation (*e.g.* G12D, G12V, or G12C) of KRAS or the Q61 mutation (*e.g.* Q61H) of KRAS. These mutations may be determined, *e.g.*, by sequencing. Further examples for a number of activating RAS mutations are also provided herein, below.

As mentioned above, in context of the present invention it has surprisingly been identified that loss of p21 increases the sensitivity of cancer cells toward treatment with the (S)-enantiomer of crizotinib. Thus, the *in vitro* method provided herein may further comprise the step of:

- (d) determining whether in said cell or tissue sample the expression and/or biological activity of p21 is reduced or absent;

wherein a cell or tissue sample which is positive for NUDT1/MTH1 and wherein the expression and/or biological activity of p21 is reduced or absent are indicative of an effective treatment and/or prevention of cancer.

In context of the present invention it has been surprisingly identified that effective treatment of cancer by using the compound for the use of the invention is independent of the patient's ALK- and/or c-Met-status. Thus, in one specific aspect, the invention relates to the above described *in vitro* method, further comprising the steps of:

- (d) determining the subject's c-Met-status; and/or
- (e) determining the subject's ALK-status;

wherein a cell or tissue sample positive for NUDT1/MTH1 and negative for c-Met is indicative of an effective treatment and/or wherein a cell or tissue sample positive for NUDT1/MTH1 and negative for ALK is indicative of an effective treatment and/or prevention of cancer.

The cell or tissue sample which is to be obtained in context of the *in vitro* method of the invention may be a cancer cell or tissue sample obtained from a cancer patient. Accordingly, a further embodiment of the invention relates to the *in vitro* method of the invention, wherein said cell or tissue sample is a cancer cell or tissue. For the determination of the status of MTH1, RAS, c-Met and/or ALK, several detection methods can be applied. Thus, one aspect of the invention relates to the *in vitro* method of the invention, wherein step (b) and/or (c) comprises at least one detection method selected from the group consisting of PCR (polymerase chain reaction), gene sequencing, ARMS (Amplification Refractory Mutation System), Peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, PCR-Invader, SNaPshot, PCR/HRMA/dHPLC, PCR/firFLP, Fluorescent In-Situ Hybridisation (FISH), Immunohistochemistry (IHC), RT-PCR, gene arrays, and gene chips. Another aspect of the invention relates to the *in vitro* method of the invention, wherein step (b), (c), (d) and/or (e) comprises at least one detection method selected from the group consisting of PCR

(polymerase chain reaction), gene sequencing, ARMS (Amplification Refractory Mutation System), Peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, PCR-Invader, SNaPshot, PCR/HRMA/dHPLC, PCR/fIRFLP, Fluorescent In-Situ Hybridisation (FISH), Immunohistochemistry (IHC), RT-PCR, gene arrays, and gene chips. These methods are well known in the art and also described herein, below.

The *in vitro* method provided herein may be performed prior to the administration of the herein described (*S*)-enantiomer of an aminoheteroaryl compound in order to evaluate whether a cancer patient will profit from the treatment with an (*S*)-enantiomer of an aminoheteroaryl compound. Accordingly, provided herein is a method of treating and/or preventing cancer in a subject in need of such treatment, wherein the method comprises:

- (i) performing the *in vitro* method as described above; and
- (ii) administering to said subject an effective amount of the (*S*)-enantiomer of an aminoheteroaryl compound provided herein if the result obtained in step (i) is indicative of an effective treatment and/or prevention of cancer.

Thus, the invention provides for a method of treating and/or preventing cancer in a subject in need of such treatment, wherein the method comprises:

- (a) obtaining a cell or tissue sample from said subject;
- (b) determining the subjects's NUDT1/MTH1-status;
- (c) optionally, determining the subject's RAS-status;
- (d) optionally, determining whether in said cell or tissue sample the expression and/or biological activity of p21 is reduced or absent;

wherein a cell or tissue sample which is positive for NUDT1/MTH1 and, optionally, which is positive for an activating RAS mutation and, optionally, wherein the expression and/or biological activity of p21 is reduced or absent, are indicative of an effective treatment and/or prevention of cancer in said subject.

The *in vitro* method of the invention may be realized by using an appropriate kit. Accordingly, another embodiment of the invention relates to a kit for carrying out the *in vitro* method of the invention, comprising polynucleotides and/or antibodies capable of detecting NUDT1/MTH1. In one aspect the invention relates to said kit, further comprising polynucleotides and/or antibodies capable of detecting RAS. The kit may additionally comprise polynucleotides and/or antibodies capable of detecting ALK and/or c-Met. The kit

may further comprise polynucleotides and/or antibodies capable of detecting p21. Several polynucleotides and antibodies for the detection of NUDT1/MTH1, RAS, ALK, c-Met and p21 are known in the art and also provided herein, below.

Accordingly, the present invention relates to a kit for carrying out the *in vitro* method for determining the effectiveness of the compound for the use of the invention, or the pharmaceutical composition of the invention, the method comprising the steps of:

- (a) obtaining a cell or tissue sample from a subject; and
- (b) determining the subject's NUDT1/MTH1-status;

wherein a NUDT1/MTH1-positive cell or tissue sample is indicative of an effective treatment and/or prevention of cancer.

The embodiments disclosed in connection with the *in vitro* method of the present invention apply, mutatis mutandis, to the kit of the present invention.

Advantageously, the kit of the present invention further comprises, optionally (a) reaction buffer(s), storage solutions, wash solutions and/or remaining reagents or materials required for the conduction of the assays as described herein. Furthermore, parts of the kit of the invention can be packaged individually in vials or bottles or in combination in containers or multicontainer units. These vials/bottles/containers or multicontainers may, in addition to the polynucleotides and/or antibodies as described herein, comprise preservatives or buffers for storage. In addition, the kit may contain instructions for use, in particular the kit may contain an instruction manual how to carry out the determination of the patient's NUDT1/MTH1-status and optionally, also the patient's RAS-, ALK-, and/or c-Met-status.

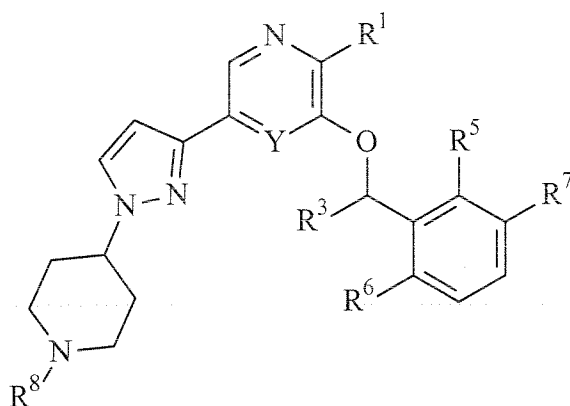
The kit of the present invention may be advantageously used, inter alia, for carrying out the *in vitro* method as described herein and/or it could be employed in a variety of further applications, e.g., as diagnostic kit, as research tool or as therapeutic tool. Additionally, the kit of the invention may contain further means for detection suitable for scientific, medical and/or diagnostic purposes. The manufacture of the kit of the present invention follows preferably standard procedures which are known to the person skilled in the art.

A further embodiment of the invention relates to the use of a kit in the *in vitro* method of the invention, the kit comprising polynucleotides and/or antibodies capable of detecting NUDT1/MTH1. The invention further relates to said use, wherein the kit further comprises polynucleotides and/or antibodies capable of detecting RAS. In addition, the invention also relates to said use, wherein the kit further comprises polynucleotides and/or antibodies

capable of detecting ALK and/or c-Met. Moreover, provided herein is the use of the herein described kit in the *in vitro* method provided herein, wherein said kit further comprises polynucleotides and/or antibodies capable of detecting p21.

In context of the present invention it has been surprisingly identified that MTH1 represents a novel target of an aminoheteroaryl compound (such as crizotinib). Furthermore, in the appended illustrative examples a screening method for identifying a target of the (*S*)-enantiomer of crizotinib is performed in SW480 cells, a colon carcinoma cell line expressing mutant KRAS. The obtained results clearly demonstrate that MTH1 (gene name NUDT1) is the main target of to (*S*)-enantiomer of crizotinib highlighting the specificity of the compound. Accordingly, the present invention further relates to a screening method for identifying a target of an aminoheteroaryl compound.

Thus, one embodiment of the invention relates to a screening method for identifying a target of an aminoheteroaryl compound having the following chemical structure represented by Formula (6),



Formula (6)

wherein:

R^1 is $-NH_2$, $-NR^2H$, $-OH$ or $-SH$;

R^2 is C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl;

R^3 is C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl or cyclopropyl;

Y is N or CR^4 ;

R^4 is hydrogen, halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl or C_{3-6} cycloalkyl;

each R^5 , R^6 and R^7 is independently fluorine, chlorine, bromine or iodine;

R^8 is hydrogen or $-A-B_n-X$, wherein

A is a single bond, $-C(=O)-$ or $-C(=O)CH_2-$;

B is C_{1-4} alkylene, C_{2-4} alkenylene or $-(OCH_2CH_2)-$;

n is 0, 1, 2, 3, 4 or 5, and

X is $-\text{NHR}^2$; $-\text{NH}_2$; $-\text{SH}$; $-\text{OH}$ or O-alkyl;

or a pharmaceutically acceptable salt, solvate or prodrug thereof;

and wherein the screening method comprises the steps of:

- (a) obtaining a cell lysate;
 - (b) contacting said aminoheteroaryl compound with said cell lysate; and
 - (c) determining whether a molecule binds to said aminoheteroaryl compound,
- wherein, if a molecule is found to bind to said aminoheteroaryl compound, then such molecule is identified as a target of said aminoheteroaryl compound.

One aspect of the invention relates to the screening method of the invention, wherein in compound of Formula (6)

R^1 is $-\text{NH}_2$, $-\text{OH}$ or $-\text{SH}$;

R^3 is methyl;

Y is CR^4 ;

R^4 is hydrogen or halogen;

each R^5 , R^6 and R^7 is independently fluorine or chlorine;

R^8 is hydrogen or $-\text{A-B}_n\text{-X}$, wherein

B is C_{1-4} alkylene or $-(\text{OCH}_2\text{CH}_2)-$;

n is 1, 2 or 3, and

X is $-\text{NH}_2$.

A further aspect of the invention relates to the screening method of the invention, wherein in compound of Formula (6)

R^8 is hydrogen;

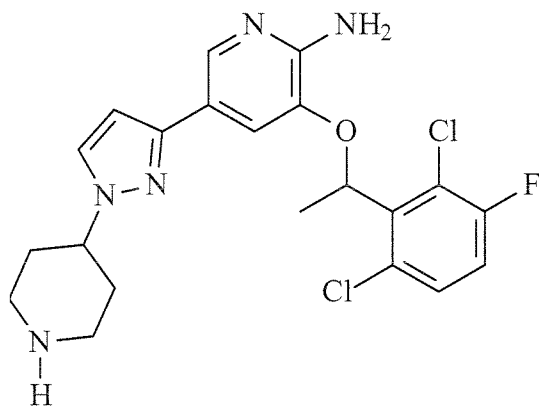
$-\text{C}(=\text{O})\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-NH}_2$;

$-\text{C}(=\text{O})\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-NH}_2$; or

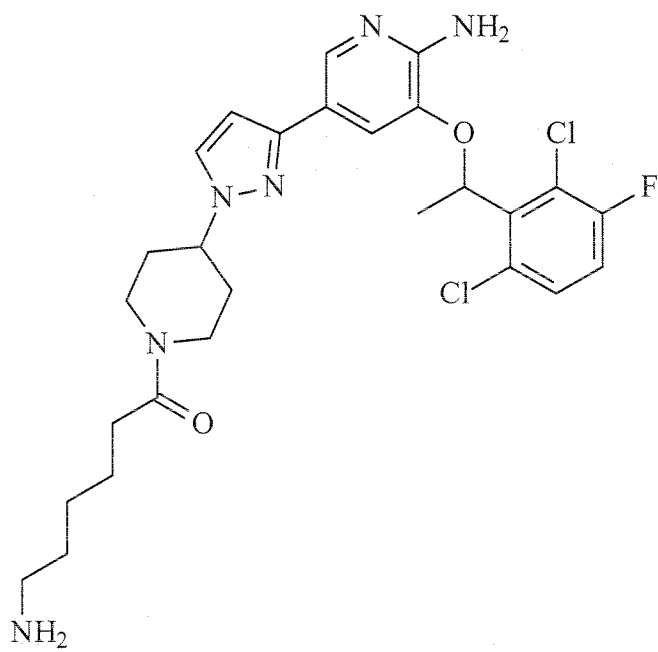
$-\text{CH}_2\text{CH}_2\text{CH}_2\text{-NH}_2$.

Another aspect of the invention relates to the screening method of the invention, wherein the compound has one of the following chemical structures represented by Formulae (7) to (10):

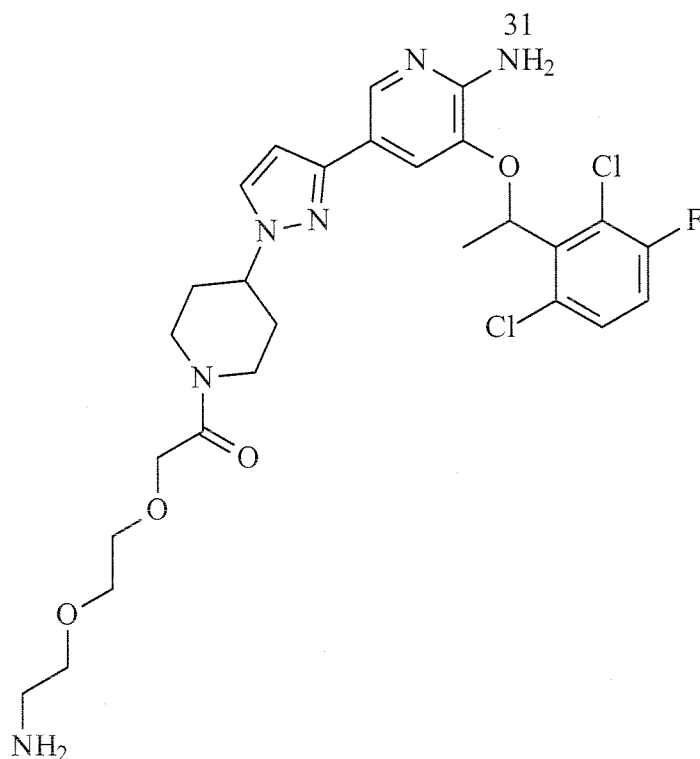
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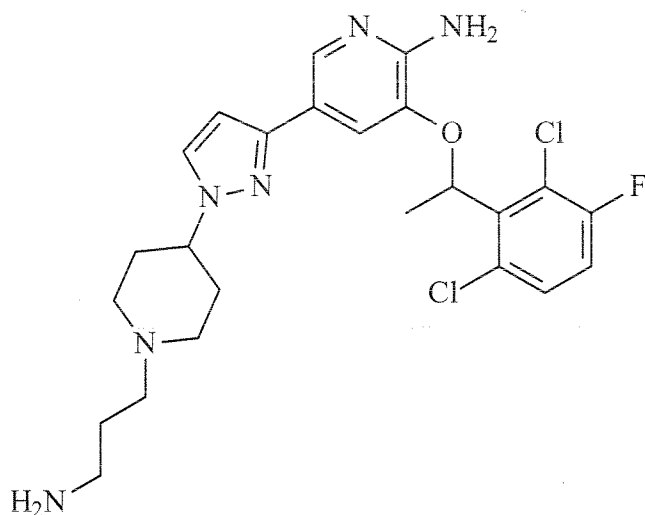
Formula (7);



Formula (8);



Formula (9);



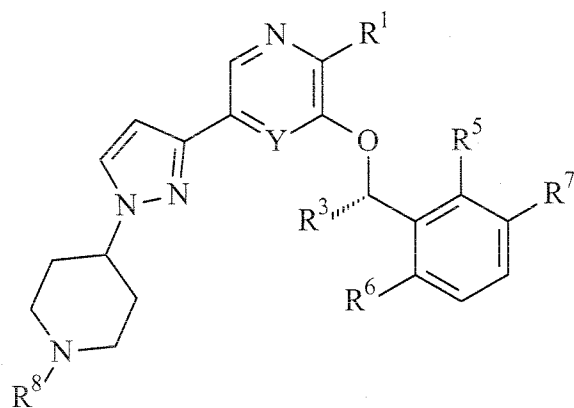
Formula (10),

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

To assess the complete interactome of the herein defined aminoheteroaryl compound considering both enantiomers, the racemic form of the aminoheteroaryl compound may be used. However, it is also envisaged to use the aminoheteroaryl compound in the (*R*)- or (*S*)-enantiomer conformation. However, in context of the invention, it is more preferred to use the (*S*)- than the (*R*)-enantiomer conformation. Accordingly, one particular embodiment of the invention is directed to the screening method of the invention, wherein said aminoheteroaryl compound is in the (*S*)-enantiomer conformation. Accordingly, said aminoheteroaryl

compound which is to be applied in the screening method of the invention may be the herein defined (*S*)-enantiomer of an aminoheteroaryl compound.

Thus, a prioritized aspect of the present invention relates to a screening method for identifying a target of an (*S*)-enantiomer of an aminoheteroaryl compound having the following chemical structure represented by Formula (1),



Formula (1)

wherein:

R^1 is $-NH_2$, $-NR^2H$, $-OH$ or $-SH$;

R^2 is C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl;

R^3 is C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl or cyclopropyl;

Y is N or CR^4 ;

R^4 is hydrogen, halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl or C_{3-6} cycloalkyl;

each R^5 , R^6 and R^7 is independently fluorine, chlorine, bromine or iodine;

R^8 is hydrogen or $-A-B_n-X$, wherein

A is a single bond, $-C(=O)-$ or $-C(=O)CH_2-$;

B is C_{1-4} alkylene, C_{2-4} alkenylene or $-(OCH_2CH_2)-$;

n is 0, 1, 2, 3, 4 or 5, and

X is $-NHR^2$; $-NH_2$; $-SH$; $-OH$ or O-alkyl;

or a pharmaceutically acceptable salt, solvate or prodrug thereof;

and wherein the screening method comprises the steps of:

- obtaining a cell lysate;
- contacting said aminoheteroaryl compound with said cell lysate; and
- determining whether a molecule binds to said aminoheteroaryl compound,

wherein, if a molecule is found to bind to said aminoheteroaryl compound, then such molecule is identified as a target of said aminoheteroaryl compound.

One embodiment of the invention relates to this screening method, wherein in compound of Formula (1)

R^1 is $-NH_2$, $-OH$ or $-SH$;

R^3 is methyl;

Y is CR^4 ;

R^4 is hydrogen or halogen;

each R^5 , R^6 and R^7 is independently fluorine or chlorine;

R^8 is hydrogen or $-A-B_n-X$, wherein

B is C_{1-4} alkylene or $-(OCH_2CH_2)-$;

n is 1, 2 or 3, and

X is $-NH_2$.

A particular embodiment of the invention is directed to this screening method, wherein in compound of Formula (1)

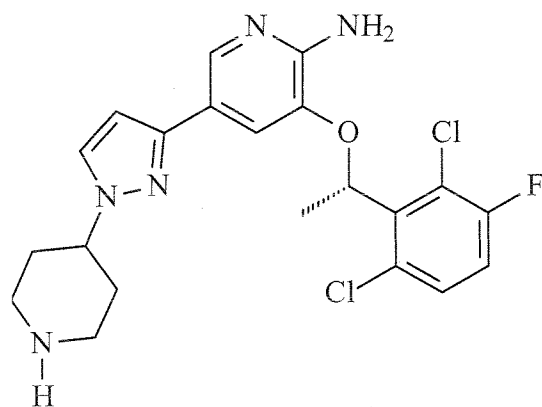
R^8 is hydrogen;

$-C(=O)CH_2CH_2CH_2CH_2CH_2-NH_2$;

$-C(=O)CH_2-O-CH_2CH_2-O-CH_2CH_2-NH_2$; or

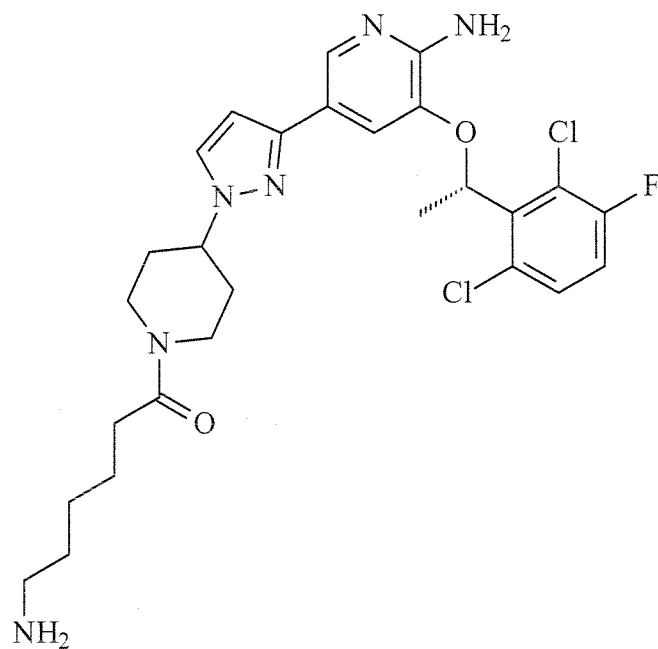
$-CH_2CH_2CH_2-NH_2$.

A prioritized aspect of the invention relates to this screening method, wherein the compound has one of the following chemical structures represented by Formulae (2) to (5):

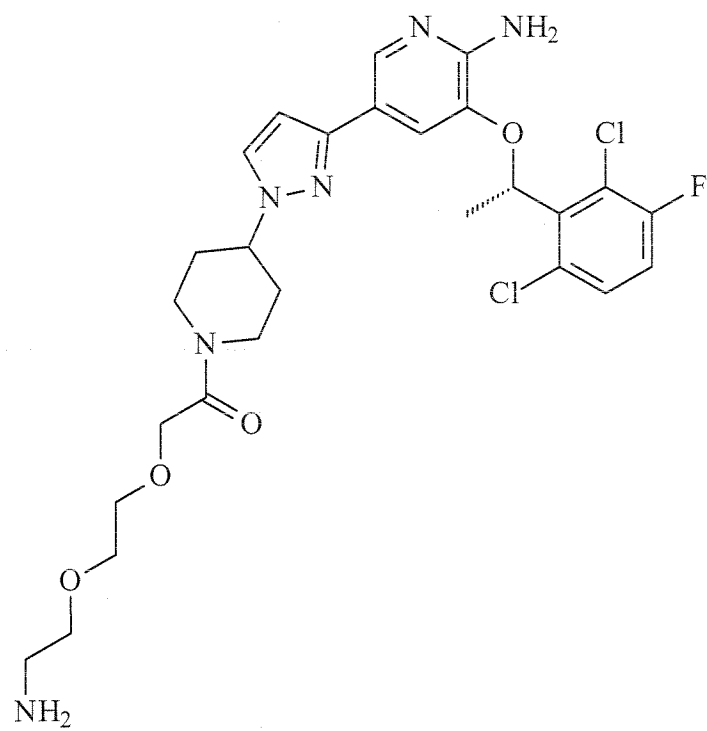


Formula (2);

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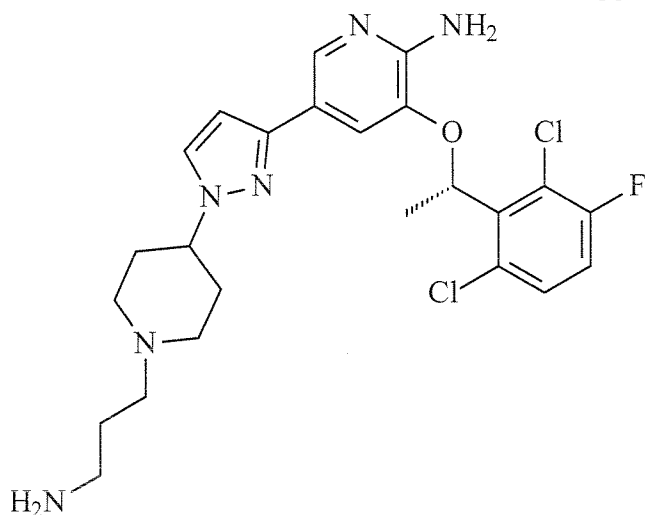


Formula (3);



Formula (4) or

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Formula (5).

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

As mentioned, the present invention relates to a screening method comprising the steps (a) to (c) as described above. A specific aspect of the invention relates to the screening method of the invention, further comprising the step of:

- (d) determining whether said (*S*)-enantiomer of an aminoheteroaryl compound alters the biological activity of said molecule,

wherein, if said (*S*)-enantiomer of an aminoheteroaryl compound is found to alter the biological activity of said molecule, then such molecule is identified as a target of said (*S*)-enantiomer of an aminoheteroaryl compound.

In context of the screening method of the invention, a cell lysate is obtained and contacted with an aminoheteroaryl compound (e.g. with the (*S*)-enantiomer of an aminoheteroaryl compound). The cells for generating this cell lysate may be cancer cells obtained from a human cancer patient. For example, said cells obtained from a human cancer patient may comprise biological material of biopsies. The meaning of “biopsies” is known in the art. For instance, biopsies comprise cancer cell(s) or cancer tissue(s) taken, e.g. by the attending physician, from a patient, suffering from cancer. Alternatively, the cells for generating the cell lysate may be obtained from (a) non-human animal(s), or from an established cancer cell line, such as an established human cancer cell line, or from an engineered cell line.

Accordingly, one embodiment of the present invention relates to the screening method of the invention, wherein said cell lysate is derived from a cell or tissue sample from a cancer patient, from an established cancer cell line or from a non-human animal. Preferably, for

carrying out the screening method of the present invention, the cell lysate is generated by using a tumour sample obtained from a human cancer patient. For example, said cells obtained from a human cancer patient may comprise biological material of biopsies. It is also preferred that the cell lysate is generated by using an established cancer cell line, such as an established human cancer cell line. For example, for generating said cell lysate, cells of the Ewing's sarcoma family of tumours (ESFT) (such as SK-ES-1 and SK-N-MC cells) may be used. In context of the screening method of the present invention, several cell lysates may be generated and analyzed in parallel. For instance, for preparing the cell lysates, human cancer cell lines with different genetic background (such as ALK-positive and ALK-negative) may be used and analyzed in parallel.

As mentioned above, within the screening method of the invention, it may be determined whether said (*S*)-enantiomer of an aminoheteroaryl compound alters the biological activity of said molecule. The biological activity of said molecule may be increased or inhibited by said (*S*)-enantiomer of an aminoheteroaryl compound. However, it is preferred that the biological activity of said molecule is inhibited. Accordingly, one aspect of the invention relates to the screening method of the invention, wherein altering the biological activity is inhibiting the biological activity.

The gist of the present invention is that an aminoheteroaryl compound, preferably the (*S*)-enantiomer of an aminoheteroaryl compound is a highly potent inhibitor of MTH1. Thus, a preferred embodiment of the invention relates to the compound for the use of the invention, the pharmaceutical composition of the invention, the *in vitro* method of the invention, the kit of the invention, the use of the invention, or the screening method of the invention, wherein said compound inhibits the biological activity of MTH1.

As mentioned above, the present invention relates to a screening method for identifying a target of an aminoheteroaryl compound. This screening method may comprise a drug pull-down assay. Therefore, a direct chemical proteomics approach in which the compound of interest (e.g. an aminoheteroaryl compound) is immobilized on sepharose beads, may be used. Such direct chemical proteomics approaches are well known in the art and described, e.g., in Superti-Furga (2012) *Designing Multi-Target Drugs*. Royal Society of Chemistry: Cambridge, 2012; p 256.⁸ An experimental set-up which may be used in context of the screening method of the present invention is provided in Figure 1. Preferably, the method

described as “Compound-centred chemical proteomics” in Figure 1 is used in context of the present invention. Also activity-based probe profiling may be applied using a modified version of one of the compounds disclosed herein.

The immobilization on sepharose beads may be achieved by a chemical reaction which establishes a covalent bond between the compound and the bead matrix. Therefore the compound requires an adequate reactive functional group for the reaction to take place which in many cases enforces chemical modification of the original compound. Of course, changing the molecular structure can alter the interaction behavior and target spectrum of the compound to be investigated. It should be noted that also the location within the molecule where the compound is immobilized can affect binding. Thus, a common practice is to check and confirm binding of already known targets for all coupleable derivatives. As illustrated in the appended examples, three derivatives of crizotinib (i.e. CeMM-144, CeMM-145 and CeMM-146 as defined herein) with different chemical linkers covering various linker lengths and chemical nature in terms of hydrophobicity have been prepared. Chemical synthesis of crizotinib derivatives CeMM-144, CeMM-145, and CeMM-146 is outlined in Figure 13. Surprisingly, as shown in the illustrative appended example, the aminopropyl-substituted CeMM-146 enriches both ALK isoforms to highest extent, followed by the PEG-derivative CeMM-145. Unmodified crizotinib and CeMM-144 bind ALK to a lesser extent than CeMM-146 and CeMM-145.

In context of the screening method of the present invention, after contacting the cell lysate with an aminoheteroaryl compound, molecules that bind to said aminoheteroaryl compound may be co-purified by affinity purification of the aminoheteroaryl compound. Methods for affinity purification are well known in the art and described, e.g., in Superti-Furga (2012) *Designing Multi-Target Drugs*. Royal Society of Chemistry: Cambridge, 2012; p 256.⁸ After purification of the molecule (e.g. the protein) that binds to said aminoheteroaryl compound, the identity of the molecule may be determined (e.g. it may be determined *which* protein bound to the aminoheteroaryl compound). For this identification step, mass spectrometry may be applied. Mass spectrometry methods are well known in the art and described, e.g., in Superti-Furga (2012) *Designing Multi-Target Drugs*. Royal Society of Chemistry: Cambridge, 2012; p 256.⁸

As reported in the prior art, MTH1 suppression was shown to cause proliferative defects in cancer cells.¹³ Thus, as indicated above, targeting MTH1 may provide a novel therapeutic

option to treat cancer. In line with this, the MTH1-status (in particular the level of MTH1 activity) may be used to monitor (i.e. accessing the effectiveness of) the treatment (i.e. the therapy) of a cancer patient. Since the activity of MTH1 may be reflected by the expression of MTH1, also the level of MTH1 expression may be used to monitor (i.e. accessing the effectiveness of) the treatment (i.e. the therapy) of a cancer patient. The treatment may comprise (an) MTH1 inhibitor(s). Thus, provided herein is a method of monitoring the effectiveness of a treatment of a cancer patient, the method comprising the following steps:

- (a) determining MTH1 activity and/or expression in a first tissue sample from said cancer patient at a first time point;
- (b) determining MTH1 activity and/or expression in a second tissue sample from said cancer patient at a second time point, and
- (c) comparing the MTH1 activity and/or expression determined in step (a) with the MTH1 activity and/or expression determined in step (b),

wherein a reduced activity and/or expression of MTH1 determined in step (b) compared to the MTH1 activity and/or expression determined in step (a) is indicative of an effective treatment, and

wherein an enhanced or identical activity and/or expression of MTH1 determined in step (b) compared to the MTH1 activity and/or expression determined in step (a) is indicative of an ineffective treatment.

The effectiveness of a treatment, in particular of a cancer therapy, may also be monitored by using a reference value. Thus, also provided herein is a method of monitoring the effectiveness of a treatment of a cancer patient, the method comprising the following steps:

- (a) determining MTH1 activity and/or expression in a tissue sample from said cancer patient, and
- (b) comparing the MTH1 activity and/or expression determined in step (a) with a reference value,

wherein a reduced activity and/or expression of MTH1 determined in step (a) compared to the reference value is indicative of an effective treatment, and

wherein an enhanced or identical activity and/or expression of MTH1 determined in step (a) compared to the reference value is indicative of an ineffective treatment.

As described herein, the methods of monitoring the effectiveness of a treatment of a cancer patient may be *in vitro* methods. In context of the above described methods of monitoring the effectiveness of a treatment of a cancer patient, said tissue sample is preferably a cancer tissue sample.

As described above, there is a strong link between MTH1 and the expression of oncogenic RAS, reactive oxygen species (ROS), oxidative damage and tumour development. Therefore, inhibitors of MTH1 represent promising agents for the treatment of cancer. Thus, provided herein is a method for identifying an anticancer substance. This method is highly useful in identifying at least one substance suspected of being an inhibitor of MTH1 activity. Potent inhibitors identified by this method can be used in the medical intervention of cancer. The method for identifying an anticancer substance comprises the steps of:

- (a) contacting MTH1 or a cell, tissue or a non-human animal comprising MTH1 with at least one test substance;
- (b) determining MTH1 activity and/or expression;
- (c) selecting a substance that decreases MTH1 activity and/or expression;

wherein the ability to decrease MTH1 activity and/or MTH1 expression is indicative for the anticancer activity of the selected substance.

Said method for identifying an anticancer substance may be characterized in that step (a) is a cell-free composition comprising the purified MTH1 protein; and step (b) comprises determining whether the at least one test substance inhibits biological activity of MTH1. The method for identifying an anticancer substance may also be characterized in that step (a) comprises a cell, tissue or non-human animal that expresses MTH1 (said expression of MTH1 may also be the expression of MTH1 in form of a transgene), and wherein step (b) comprises determining whether the at least one test substance inhibits biological activity and/or expression of MTH1. Step (b) of the method for identifying an anticancer substance may comprise detecting a decrease in MTH1 biological activity and/or a decrease of MTH1 expression within a cell lysate derived from a cell, tissue or non-human animal.

Most of the MTH1 which is applied in step (a) of the method for identifying an anticancer substance is considered to be functional, i.e. to have the ability to hydrolyse oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP. It is envisaged, that at least 25% or 50%, preferably at least 50%, 75% or 90%, and more preferably at least 95%, 98% or 99% of the MTH1 which is applied in step (a) is functional.

It is noted that in step (a) of the method for identifying an anticancer substance, the term "contacting MTH1 or a cell, tissue or a non-human animal comprising MTH1 with at least one test substance" also relates to "contacting a derivative of MTH1 or a functional fragment of MTH1 or a cell, tissue or a non-human animal comprising a derivative of MTH1 or a

functional fragment of MTH1 with at least one test substance". A definition of the terms "derivative of MTH1" and "functional fragment of MTH1" is provided herein, below.

In accordance with the method for identifying an anticancer substance described herein, at least one test substance is contacted with a cell, tissue or non-human animal comprising MTH1. For example said cell, tissue or non-human animal may express a MTH1 gene, in particular also (an) additional (copy) copies of a MTH1 gene, (a) MTH1 mutated gene(s), a recombinant MTH1 gene construct and the like. The ability of a test substance to decrease MTH1 activity and/or expression may, accordingly, be determined by measuring the expression level (e.g. mRNA or protein) of such gene products of MTH1 or of corresponding gene constructs, wherein a low expression level (compared to a standard or reference value) is indicative for the ability of the test substance to decrease MTH1 activity and/or expression.

As described herein, in one aspect of the method for identifying an anticancer substance, said cell, tissue or non-human animal is genetically modified. Said cell, tissue or non-human animal may comprise a reporter gene expression construct. In particular, said reporter gene expression construct may comprise the MTH1-promoter and/or enhancer or an MTH1-dependent promoter and/or an enhancer of an MTH1-dependent factor linked to a reporter gene. A MTH1-dependent promoter (i.e. the promoter of an MTH1-dependent factor) may be the promoter of a protein that acts downstream of MTH1. For example, said protein may be transcriptionally upregulated or downregulated in response to hydrolysis of oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP. Accordingly, if said protein is transcriptionally upregulated in response to MTH1 enzymatic activity, a reduced transcription of the reporter gene construct would be indicative for a decreased MTH1 activity and/or expression. On the other hand, if said factor is transcriptionally downregulated in response to MTH1 enzymatic activity, an enhanced transcription of the reporter gene construct would be indicative for decreased MTH1 activity and/or expression. Accordingly, as defined and disclosed herein, the term "comprising MTH1" refers not only to the MTH1 gene(s) or protein(s) known in the art and described herein. Also reporter constructs comprising a promoter and/or enhancer region of MTH1 can be used in the method for identifying an anticancer substance. Accordingly, the cell(s), tissue(s) and/or non-human animal(s) used in the context of the method for identifying an anticancer substance can comprise reporter constructs. Exemplary reporters are luciferase and fluorescent proteins, like GFP, RFP and the like. Also reporter constructs comprising a promoter and/or enhancer region of MTH1 (or of MTH1-dependent factors) can be used. Accordingly, the cell(s), tissue(s) and/or non-human

animal(s) used in the context of the present invention, can be stably or transiently transfected with reporter constructs.

The used non-human animal or cell may be transgenic or non transgenic. Within said transgenic cell or non-human animal, at least one MTH1 gene may be overexpressed, thus the MTH1 activity in the non-human transgenic animal or transgenic cell may be enhanced. Generally, it is preferred herein that MTH1 is highly expressed in (a) cell(s), tissue(s), non-human animal(s) to be used in the method for identifying an anticancer substance as described herein. The term “transgenic non-human-animal”, “transgenic cell” or “transgenic tissue” as used herein refers to a non-human animal, tissue or cell, that comprises different genetic material. The term “genetic material” in this context may be any kind of a nucleic acid molecule, or analogues thereof. The term “different” means that additional or fewer genetic material in comparison to the genome of the wild type animal or cell. An overview of different expression systems to be used for generating a transgenic cell/animal refers for example to Methods in Enzymology 153 (1987), 385-516, in Bitter *et al.* (Methods in Enzymology 153 (1987), 516-544) and in Sawers *et al.* (Applied Microbiology and Biotechnology 46 (1996), 1-9), Billman-Jacobe (Current Opinion in Biotechnology 7 (1996), 500-4), Hockney (Trends in Biotechnology 12 (1994), 456-463), Griffiths *et al.*, (Methods in Molecular Biology 75 (1997), 427-440).

Non-limiting examples of the (transgenic) non-human animals or derived (transgenic) cells are selected from the group consisting of a mouse, a rat, a rabbit, a guinea pig and *Drosophila*. In a preferred embodiment, the (transgenic) non-human animal or (transgenic) cell is or is derived from a mammal. Generally, the (transgenic) cell may be a eukaryotic cell. For example, the (transgenic) cell in accordance with the present invention may be but is not limited to yeast, fungus, plant or animal cell. The (transgenic) cell may be derived from human, e.g., from human cancer tissue. The (transgenic) cell may be an established cancer cell line. In general, the transformation or genetically engineering of a cell with a nucleic acid construct or a vector can be carried out by standard methods, as for instance described in Sambrook and Russell (2001), Molecular Cloning: A Laboratory Manual, CSH Press, Cold Spring Harbor, NY, USA; Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, 1990.

In accordance with the method for identifying an anticancer substance, the determined activity and/or expression of MTH1 may be compared to a standard or reference value of MTH1

activity and/or expression, respectively. The standard/reference value may be determined in a cell, tissue, or non-human animal, which has not been contacted with a test substance. The decrease in the activity and/or expression of MTH1 may also be compared to the decrease in MTH1 activity and/or expression by (a) routinely used reference substance(s). A skilled person is easily in the position to determine/assess whether the activity and/or expression of MTH1 is (preferably statistically significant) decreased.

In context of the method for identifying an anticancer substance, the term “contacting” refers to the addition of at least one test substance to MTH1, or to a cell, tissue, or non-human animal comprising MTH1. The term “contacting” also refers to the addition of a test substance to a cell comprising MTH1 in a way that the test substance may become effective to the cell upon cellular uptake and thereby exerts its inhibitory function on MTH1.

The term “test substance” as used herein refers to a molecule or substance or composition or agent or any combination thereof to be tested by the method for identifying an anticancer substance. A test substance can, in principle, be obtained from any source. The at least one test substance may be a naturally occurring substance or a substance produced by a transgenic organism and optionally purified to a certain degree and/or further modified. Practically, the test substance may be taken from a compound library.

The test substance may be a potential inhibitor of activity and/or expression of MTH1. A test substance can be any chemical, such as an inorganic chemical, an organic chemical, a protein, a peptide, a carbohydrate, a lipid, an siRNA against MTH1, an shRNA against MTH1, or a combination thereof or any of the compounds or compositions described herein. A test substance to be used herein may be, inter alia, a substance or composition which is of chemical or biological origin, which is naturally occurring and/or which is synthetically, recombinantly and/or chemically produced. Thus, a test substance may be a protein, protein-fragment, peptide, amino acid and/or derivatives thereof or another substance, which binds to and/or interacts with MTH1, a regulatory protein/sequence of MTH1 function or functional fragments thereof. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, N.J.), Brandon Associates (Merrimack, N.H.), and Microsource (New Milford, Conn.). A rare chemical library is available from Aldrich (Milwaukee, Wis.). Alternatively, libraries of natural substances in the form of bacterial, fungal, plant and animal extracts are available from e.g. Pan Laboratories (Bothell, Wash.) or MycoSearch (N.C.) are readily producible. Additionally, natural and

synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. Results obtained from deorphanisation programs based on phylogenetic analysis methods may aid to find natural factors that bind to or interact with MTH1 and, e.g., will allow in silico profiling of substances which potentially have the ability to decrease MTH1 activity and/or expression. Factors that bind to or interact with MTH1 may be inhibitors of MTH1 and thus, may be anticancer substances.

The generation of chemical libraries with potential factors that bind to or interact with MTH1 is well known in the art. For example, combinatorial chemistry may be used to generate a library of substances. A combinatorial chemical library is a collection of diverse chemical substances generated by either chemical synthesis or biological synthesis by combining a number of chemical "building block" reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining amino acids in every possible combination to yield peptides of a given length. Millions of chemical substances can theoretically be synthesized through such combinatorial mixings of chemical building blocks. For example, one commentator observed that the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds (see, e.g., Gallop, *Journal of Medicinal Chemistry*, Vol. 37, No. 9, 1233-1250 (1994)). Other chemical libraries known to those in the art may also be used, including natural product libraries. Once generated, combinatorial libraries are screened for substances that possess desirable biological properties. For example, substances which may be useful as drugs or to develop drugs would likely have the ability to bind to the target protein (e.g. MTH1).

In addition, in the context of the present invention, libraries of substances may be screened to identify substances that may function as an antagonist or inhibitor of MTH1. First, a library of small molecules may be generated using methods of combinatorial library formation well known in the art. US 5,463,564 and US 5,574,656 are two such teachings. Then the library substances may be screened to identify those substances that possess desired structural and functional properties. Methods for screening libraries are well known in the art and discussed, e.g., in US 5,684,711. In addition, a wide variety of screening techniques are known for a large number of naturally-occurring targets when the biochemical function of the target protein is known. For example, some techniques involve the generation and use of small peptides to probe and analyze target proteins both biochemically and genetically in order to identify and develop drug leads. Such techniques include the methods described in WO 99/35494, WO 98/19162 and WO 99/54728.

Test substances may encompass numerous chemical classes, preferably they are organic compounds, and more preferably small (organic) molecules, such as the herein defined aminoheteroaryl compounds.

Test substances may comprise functional groups necessary for structural interaction with a protein (in particular with MTH1), particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The test substances often comprise carbocyclic or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. The test substance and/or the selected anticancer substance may be modified to enhance efficacy, stability, pharmaceutical compatibility, and the like. Structural identification of an anticancer substance may be used to identify, generate, or screen additional anticancer substances.

In accordance with the herein described method for identifying an anticancer substance, the substances selected in the first screen may be subject to subsequent screens in order to verify the previous findings and to select the most potent inhibitors/antagonists of MTH1. Upon multiple screening and selection rounds those substances will be selected which show a pronounced capacity to inhibit/antagonize MTH1.

The term “decreases MTH1 activity and/or expression” in step (c) of the method for identifying an anticancer substance means that the “activity and/or expression of MTH1” is reduced upon contacting MTH1 or a cell, tissue, or non-human animal comprising MTH1 with the at least one test substance, preferably in comparison to a (control) standard or reference value, wherein a decrease of the MTH1 activity and/or expression is indicative for the anticancer activity of the selected substance (i.e. for the capacity of the selected substance to ameliorate cancer).

As described herein a substance that “decreases MTH1 activity” relates to substance that decreases the biological activity of MTH1 (i.e. the ability to hydrolyse oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP). Methods for measuring the MTH1 biological activity are known in the art and also provided herein. In addition, a substance that “decreases MTH1 expression” relates to a decreased expression of the gene(s) encoding the MTH1 protein(s). Therefore, a quantitative assessment of the gene product (e.g. protein or spliced, unspliced or partially spliced mRNA) can be performed in order to evaluate decreased expression of the gene(s) encoding the MTH1 protein(s). The person skilled in the art is aware of standard methods to be used in this context or may deduce these methods from standard textbooks (e.g.

Sambrook, Russell "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory, N.Y. (2001)). For example, quantitative data on the respective concentration/amounts of mRNA from MTH1 can be obtained by Northern Blot, Real Time PCR and the like.

Preferably, the MTH1 activity and/or expression may be decreased by at least about 10 %, 20 %, 30 %, 40 %, preferably by at least 50 %, 60 %, 70 %, 80 %, 90 %, or 100 % compared to a control sample. As mentioned, a person skilled in the art is aware of standard methods to be used for determining or quantitating activity of MTH1 or expression of a nucleic acid molecule encoding MTH1 (or fragments thereof). In addition, a method for determining and quantitating activity of MTH1 is described in detail herein and in the appended examples. In addition, the expression of MTH1 can be determined on the protein level by taking advantage of immunoagglutination, immunoprecipitation (e.g. immunodiffusion, immunoelectrophoresis, immune fixation), western blotting techniques (e.g. (in situ) immuno histochemistry, (in situ) immuno cytochemistry, affinitychromatography, enzyme immunoassays), and the like. Amounts of purified polypeptide in solution can be determined by physical methods, e.g. photometry. Methods of quantifying a particular polypeptide in a mixture rely on specific binding, e.g. of antibodies. Specific detection and quantitation methods exploiting the specificity of antibodies comprise for example immunohistochemistry (in situ). For example, concentration/amount of MTH1 proteins in a cell, tissue or a non-human animal can be determined by enzyme linked-immunosorbent assay (ELISA). Alternatively, Western Blot analysis or immunohistochemical staining can be performed. Western blotting combines separation of a mixture of proteins by electrophoresis and specific detection with antibodies. Electrophoresis may be multi-dimensional such as 2D electrophoresis. Usually, polypeptides are separated in 2D electrophoresis by their apparent molecular weight along one dimension and by their isoelectric point along the other direction.

Expression can also be determined on the nucleic acid level (e.g. if the gene product/product of the coding nucleic acid sequence is an unspliced/partially spliced/spliced mRNA) by taking advantage of Northern blotting techniques or PCR techniques, like in-situ PCR or Real time PCR. Quantitative determination of mRNA can be performed by taking advantage of northern blotting techniques, hybridization on microarrays or DNA chips equipped with one or more probes or probe sets specific for mRNA transcripts or PCR techniques referred to above, like, for example, quantitative PCR techniques, such as Real time PCR. These and other suitable methods for detection and/or determination of the concentration/amount of (specific) mRNA

or protein(s)/polypeptide(s) are well known in the art and are, for example, described in Sambrook (2001), loc. cit.).

A skilled person is capable of determining the amount of mRNA or polypeptides/proteins, in particular the gene products described herein above, by taking advantage of a correlation, preferably a linear correlation, between the intensity of a detection signal and the amount of, for example, the mRNA or polypeptides/proteins to be determined.

In accordance with the method for identifying an anticancer substance, the amount of decrease of MTH1 activity and/or expression may be statistically significant and a substance may be selected, if the MTH1 activity and/or expression (or of a corresponding reporter signal) is strongly decreased, preferably is very low or non-detectable. For example, the MTH1 activity and/or expression (or of a corresponding reporter signal) may be decreased by at least 50%, 60%, 70%, 80%, more preferably by at least 90% compared to the (control) standard value.

As used herein, in particular in the context of the embodiments relating to formulae (1) and (6), the following definitions apply:

The term "halogen" includes fluorine, chlorine, bromine and iodine.

C₁₋₆ alkyl refers to straight or branched alkyl. Examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, hexyl, isohexyl. Accordingly, C₁₋₃ alkyl refers to straight or branched alkyl, and for example, methyl, ethyl, n-propyl, isopropyl.

C₂₋₆ alkenyl refers to straight or branched alkenyl having one or more double bond(s) at any position thereof. Examples include vinyl, allyl, propenyl, isopropenyl, butenyl, isobutenyl, prenyl, butadienyl, pentenyl, isopentenyl, pentadienyl, hexenyl, isohexenyl, hexadienyl. Accordingly, C₂₋₃ alkenyl refers to straight or branched alkenyl having one or more double bond(s) at any position thereof. Examples include vinyl, allyl, propenyl, isopropenyl.

C₂₋₆ alkynyl refers to straight or branched alkynyl having one or more triple bond(s) at any position thereof. Examples include ethynyl, propynyl, butynyl, pentynyl, hexynyl. Alkynyl groups may additionally have a double bond at any position thereof. Accordingly, C₂₋₃

alkynyl refers to straight or branched alkynyl having one or more triple bond(s) at any position thereof. Examples include ethynyl and propynyl.

C₁₋₄ alkylene refers to a linear divalent hydrocarbon chain. Examples include methylene, ethylene, propylene and butylene.

C₂₋₄ alkenylene refers to a linear divalent hydrocarbon chain having one or more double bonds at any position thereof, and includes, for example, vinylene, propenylene and butenylene.

In accordance with the present invention, the terms “(S)-enantiomer of an aminoheteroaryl compound of the invention”, “(S)-enantiomer of an aminoheteroaryl compound for the use of the invention” and “compound for the use of the invention” relate to the herein defined “(S)-enantiomer of an aminoheteroaryl compound”. In addition, as used herein, the terms “(S)-enantiomer of an aminoheteroaryl compound of the invention”, “(S)-enantiomer of an aminoheteroaryl compound for the use of the invention” and “compound for the use of the invention” are synonyms for the “(S)-enantiomer of an aminoheteroaryl compound” which is shown in any one of Formulae (1) to (5) and described in claim 1 of the appended claims. Preferably, the terms “(S)-enantiomer of an aminoheteroaryl compound of the invention”, “(S)-enantiomer of an aminoheteroaryl compound for the use of the invention” and “compound for the use of the invention” are synonyms for the “(S)-enantiomer of an aminoheteroaryl compound” which is described in items 1 to 4, 8 to 20 and 37.

As demonstrated herein, in contrast to (R)-crizotinib, the “(S)-enantiomer of an aminoheteroaryl compound for the use of the invention” is not restricted to the use in treating ALK-positive cancer. Thus, the “(S)-enantiomer of an aminoheteroaryl compound for the use of the invention” can also be used to treat ALK-negative cancer. Accordingly, the terms “(S)-enantiomer of an aminoheteroaryl compound of the invention”, “(S)-enantiomer of an aminoheteroaryl compound for the use of the invention” and “compound for the use of the invention” further relate to “an (S)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing cancer in a subject, wherein the treatment and/or prevention of cancer is independent of the ALK-status and/or the c-Met-status of the cancer cell or tissue of said subject.

More specifically, the terms “(S)-enantiomer of an aminoheteroaryl compound of the invention”, “(S)-enantiomer of an aminoheteroaryl compound for the use of the invention”, and “compound for the use of the invention” relate to a compound which has the ability to

inhibit the biological activity (in particular the catalytic activity) of MTH1. Accordingly, the terms “(*S*)-enantiomer of an aminoheteroaryl compound of the invention”, “(*S*)-enantiomer of an aminoheteroaryl compound for the use of the invention” and “compound for the use of the invention” relate to an (*S*)-enantiomer of an aminoheteroaryl compound which is capable of inhibiting the biological activity of MTH1 for use in treating and/or preventing cancer in a subject.

As mentioned above, the “(*S*)-enantiomer of an aminoheteroaryl compound of the invention” is not restricted to the use in treating ALK-positive cancer. Thus, the terms “(*S*)-enantiomer of an aminoheteroaryl compound of the invention”, “(*S*)-enantiomer of an aminoheteroaryl compound for the use of the invention” and “compound for the use of the invention” further relate to an (*S*)-enantiomer of an aminoheteroaryl compound which has the ability to inhibit the biological activity of MTH1 for use in treating and/or preventing cancer in a subject, wherein the treatment and/or prevention of cancer is independent of the ALK-status and/or c-Met-status of the cancer cell or tissue of said subject. Means and methods for determining whether a particular compound has the ability to inhibit the biological activity of MTH1 are known in the art and provided herein, below.

The “(*S*)-enantiomer of an aminoheteroaryl compound for the use of the invention” may be the (*S*)-enantiomer of a derivative of crizotinib (such as CeMM-144, CeMM-145 or CeMM-146). Preferably, the “(*S*)-enantiomer of an aminoheteroaryl compound for the use of the invention” is CeMM-146. More preferably, the “(*S*)-enantiomer of an aminoheteroaryl compound for the use of the invention” is (*S*)-crizotinib (i.e. the (*S*)-enantiomer of the chemical substance 3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine).

The person skilled in the art has the ability to synthesize the (*S*)-enantiomer of crizotinib (i.e. the (*S*)-enantiomer of the chemical substance 3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine). In addition, the preparation of the (*S*)-enantiomer of crizotinib (i.e. the (*S*)-enantiomer of the chemical substance 3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine) is described herein in Figure 14. The synthetic strategy depends on the use of optically pure starting materials and may follow previously published procedures for synthesis of racemic and/or (*R*)-crizotinib. Procedures for synthesis of racemic and/or (*R*)-crizotinib are given in, e.g., de Koning (2011) Organic Process Research & Development, 15: 1018-1026³⁸, which is herein incorporated by reference in its entirety.

A “functional fragment” of MTH1 is a polypeptide comprising a fragment of MTH1 and having the biological activity of MTH1. As described herein above and below, the biological activity of MTH1 is the catalytic activity of MTH1, i.e. the ability to hydrolyse oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP. A functional fragment of MTH1 may be a polypeptide comprising a fragment of one of the amino acid sequences of MTH1 as provided herein as SEQ ID Nos. 2, 4, 6, 8, 10, 12, 14 to 16. A functional fragment of MTH1 may also be a polypeptide which is encoded by a fragment of one of the nucleotide sequences of MTH1 as provided herein as SEQ ID Nos. 1, 3, 5, 7, 9, 11 and 13. A functional fragment of MTH1 may be a polypeptide comprising at least 50%, preferably at least 60%, more preferably at least 70%, at least 80%, or at least 90%, and most preferably at least 95% or at least 99 % of one of the amino acid sequences of MTH1 as provided herein as SEQ ID Nos. 2, 4, 6, 8, 10, 12, 14 to 16. A functional fragment of MTH1 may also be a polypeptide which is encoded by a nucleotide sequence comprising at least 50%, preferably at least 60%, more preferably at least 70%, at least 80%, or at least 90%, and most preferably at least 95% or at least 99% of one of the nucleotide sequences of MTH1 as provided herein as SEQ ID Nos. 1, 3, 5, 7, 9, 11 and 13.

A “derivative” of MTH1 is a polypeptide having homology to MTH1 and having the biological activity of MTH1. A derivative of MTH1 may be a polypeptide having at least 60%, preferably at least 70% or at least 80%, more preferably at least 90% and most preferably at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to one of the amino acid sequences of MTH1 as provided herein as SEQ ID Nos. 2, 4, 6, 8, 10, 12, 14 to 16. A derivative of MTH1 may also be a polypeptide encoded by a nucleotide sequence having at least 60%, preferably at least 70% or at least 80%, more preferably at least 90% and most preferably at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to one of the nucleotide sequences of MTH1 as provided herein as SEQ ID Nos. 1, 3, 5, 7, 9, 11 and 13. As described herein above and below, the biological activity of MTH1 is the catalytic activity of MTH1, i.e. the ability to hydrolyse oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP.

In context of the present invention, the term “having homology to”, means that the respective amino acid or nucleotide sequences have identities of at least 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% to the sequences shown herein, e.g. those of MTH1, wherein the higher identity values are preferred upon the lower ones. In accordance with the present invention, the term “identity/identities” or “percent identity/identities” in the context of two or more nucleic acid or amino acid sequences, refers to two or more sequences or subsequences

that are the same, or that have a specified percentage of amino acid residues or nucleotides that are the same (e.g., 60% or 70% identity, preferably, 70-95% identity, more preferably at least 95%, 96%, 97%, 98% or 99% identity with the nucleotide sequences of, e.g., SEQ ID Nos: 1, 3, 5, 7, 9, 11 and 13, or with the amino acid sequences of, e.g., SEQ ID Nos: 2, 4, 6, 8, 10, 12, 14 to 16), when compared and aligned for maximum correspondence over a window of comparison, or over a designated region as measured using a sequence comparison algorithm as known in the art, or by manual alignment and visual inspection.

Preferably the described identity exists over a region that is at least about 25 to 75 amino acids or nucleotides in length. It is more preferred that the described identity exists over a region that is about 75 to 150 amino acids or nucleotides in length. In case of nucleotide sequences, the described identity most preferably exists over a region that is at least about 75 to 225, and more preferred over a region that is at least about 225 to 450 nucleotides in length. Those having skill in the art will know how to determine percent identity between/among sequences using, for example, algorithms such as those based on CLUSTALW computer program (Thompson; 1994; Nucl Acids Res; 2; 4673-4680) or FASTDB (Brutlag; 1990; Comp App Biosci; 6; 237-245), as known in the art.

In accordance with the present invention it is envisaged that the (*S*)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing ALK-negative cancer, has the ability to inhibit the biological activity of MTH1.

The term "biological activity" as used herein relates to the functionality of a molecule (e.g. a polypeptide such as MTH1). A molecule (e.g. a polypeptide) is "functional" means, in context of the invention, that the molecule (e.g. the polypeptide) has the ability to carry out a specific "function". Accordingly, the term "biological activity" relates to the ability of a molecule (e.g. of a specific protein such as MTH1) to carry out a specific function. For instance, in context of the present invention, the biological activity of MTH1 comprises the catalytic activity of MTH1, *i.e.*, the ability to hydrolyse oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP, yielding the corresponding monophosphate and pyrophosphate (PPi). To analyse whether a (*S*)-enantiomer of an aminoheteroaryl compound has the ability to alter (e.g. to inhibit) the biological activity of MTH1, an enzymatic assay using recombinant MTH1 can be performed. For instance a luminescence-based assay which monitors the production of PPi generated by MTH1-mediated 8-oxo-dGTP hydrolysis may be used following a protocol which has been used to determine MTH1 enzyme kinetics (see, e.g. Svensson (2011) FEBS Lett. 585: 2617-2621.¹⁵

More specifically, for determining the ability of a compound in inhibiting biological activity of MTH1, MTH1 may be expressed in an appropriate host cell (such as a bacterial cell) and MTH1 may be purified. Subsequently, the purified MTH1 may be incubated with the (*S*)-enantiomer of an aminoheteroaryl compound of interest. The activity of MTH1 can be monitored by measuring the production of PPi generated by MTH1-mediated 8-oxo-dGTP hydrolysis.

Additionally, in context of the present invention, an “enhanced”, or “high” biological activity of a molecule (e.g. of a protein such as MTH1) means that the biological activity of the molecule (e.g. of MTH1) is enhanced compared to a control. In line with this, in context of the present invention, an “inhibited”, “reduced”, “low” or “less” biological activity of a molecule (e.g. of MTH1) means that the biological activity of the molecule (e.g. of MTH1) is reduced compared to a control. For example, the reason for a reduced biological activity of a molecule (e.g. of a protein such as MTH1) compared to a control may be the presence of a compound (e.g. an (*S*)-enantiomer of an aminoheteroaryl compound) that inhibits the biological activity of the molecule (e.g. of MTH1). In this respect, the control could be the same sample but without the compound that inhibits the biological activity of the molecule.

The pharmaceutical composition described herein can be formulated by techniques known to the person skilled in the art, such as the techniques published in Remington's Pharmaceutical Sciences, 20th Edition. The pharmaceutical composition can be formulated as dosage forms for, e.g. oral administration. However, also parenteral, such as intramuscular, intravenous, subcutaneous, intradermal, intraarterial, rectal, nasal, topical or vaginal administration is envisaged. In context of the present invention, the most preferred route of administration of the herein defined (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) is oral administration.

Dosage forms for oral administration include coated and uncoated tablets, soft gelatine capsules, hard gelatine capsules, lozenges, troches, solutions, emulsions, suspensions, syrups, elixiers, powders and granules for reconstitution, dispersible powders and granules, medicated gums, chewing tablets and effervescent tablets.

Dosage forms for parenteral administration include solutions, emulsions, suspensions, dispersions, powders and granules for reconstitution. Emulsions are a preferred dosage form for parenteral administration. Dosage forms for rectal and vaginal administration include suppositories and ovula. Dosage forms for nasal administration can be administered via inhalation and insufflation, for example by a metered inhaler.

Pharmaceutically acceptable salts of compounds that can be used in the present invention can be formed with various organic and inorganic acids and bases. Exemplary acid addition salts comprise acetate, adipate, alginate, ascorbate, benzoate, benzenesulfonate, hydrogensulfate, borate, butyrate, citrate, caphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oxalate, pectinate, persulfate, 3-phenylsulfonate, phosphate, picate, pivalate, propionate, salicylate, sulfate, sulfonate, tartrate, thiocyanate, toluenesulfonate, such as tosylate, undecanoate and the like. Exemplary base addition salts comprise ammonium salts, alkali metal salts, such as sodium, lithium and potassium salts; earth alkali metal salts, such as calcium and magnesium salts; salts with organic bases (such as organic amines), such as benzazethine, dicyclohexylamine, hydrabine, N-methyl-D-glucamine, N-methyl-D-glucamide, t-butylamine, salts with amino acids, such as arginine, lysine and the like.

Pharmaceutically acceptable solvates of compounds that can be used in the present invention may exist in the form of solvates with water, for example hydrates, or with organic solvents such as methanol, ethanol or acetonitrile, i.e. as a methanolate, ethanolate or acetonitrilate, respectively.

Pharmaceutically acceptable prodrugs of compounds that can be used in the present invention are derivatives which have chemically or metabolically cleavable groups and become, by solvolysis or under physiological conditions, the compounds of the invention which are pharmaceutically active in vivo. The prodrug derivative form often offers advantages of solubility, tissue compatibility or delayed release in a mammalian organism (see, Bundgaard, H., Design of Prodrugs, pp. 7-9, 21-24, Elsevier, Amsterdam 1985).

These pharmaceutical compositions described herein can be administered to the subject at a suitable dose. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Generally, the regimen as a regular administration of the pharmaceutical composition comprising the herein defined (*S*)-enantiomer of an aminoheteroaryl compound should be in the range of 0,1 µg to 5000 mg units per day, in

some embodiments 0,1 µg to 1000 mg units per day. If the regimen is a orally administration, it may be in the range of 100 mg units per day to 5000 mg units per day, preferably 500 mg units per day. This 500 mg units per day may be administrated as 250 mg dosages twice daily. If the regimen is a continuous infusion, it may also be in the range of 0,1 ng to 10 µg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. It is also envisaged to use regimens which provide for escalating doses. It is in particular preferred that the patient in need of the medical intervention as provided herein receives high dosages of the herein defined (*S*)-enantiomer of an aminoheteroaryl compound. Such high dosages may comprise between 1 to 500 mg/kg, however, also other dosage regimens are envisaged and can be attended to by the attending physician. As shown in the appended examples, in a xenograft mouse study, 25 mg/kg body weight of the (*S*)-enantiomer of crizotinib was well-tolerated and led to a significant reduction of tumour volume of more than 50%. Therefore, the herein defined (*S*)-enantiomer of an aminoheteroaryl compound may be administered in a dosage of 25 mg/kg body weight (e.g. when administered to animals such as mice). There is further provided a regimen as a regular administration of the pharmaceutical composition comprising the herein defined (*S*)-enantiomer of an aminoheteroaryl compound in combination with chemotherapy, preferably with a PARP inhibitor and/or an EGFR inhibitor wherein said combined preparation is for simultaneous, separate or sequential use. It is also envisaged in context of the present invention to administer the pharmaceutical composition comprising the herein defined (*S*)-enantiomer of an aminoheteroaryl compound in combination with radiotherapy, wherein said combined administration is a simultaneous, separate or sequential administration.

In the context of the present invention, the following modes of administration of the (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib), alone or in combination with radiotherapy and/or chemotherapy (such as a PARP inhibitor and/or an EGFR inhibitor), are preferred:

- Oral administration in constant or escalating doses;
- Subcutaneous administration in constant or escalating doses; and/or
- Intravenous administration in constant or escalating doses.

The most preferred mode of administration is oral administration in constant doses.

As described herein above and below, the invention provides for a method of treating and/or preventing cancer in a subject in need of such treatment, wherein the method comprises administering an effective amount of the (*S*)-enantiomer of an aminoheteroaryl compound provided herein or the pharmaceutical composition provided herein to said subject. One aspect of the present invention relates to the method of treatment and/or prevention of cancer in a subject, wherein the treatment and/or prevention is independent of the ALK-status and/or the c-Met-status of the cancer cell or tissue of said subject, comprising administering to said subject a therapeutically effective amount of the herein defined (*S*)-enantiomer of an aminoheteroaryl compound. Thus, the cancer to be treated and/or prevented may be ALK-negative (i.e. may not have an activating ALK aberration) and/or may be c-Met-negative.

Accordingly, the present invention provides for means and methods for the treatment of subjects in need of such a treatment (either curative or preventive) which suffer from cancer. This cancer may be an ALK-negative cancer, (i.e. a cancer wherein the cancer cells or cancer tissue do not have an activating ALK aberration) and/or a c-Met-negative cancer. The method of treatment of such disorder comprises the administration of a pharmaceutically active amount of a herein defined (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) alone or in combination with radiotherapy and/or chemotherapy (such as a PARP inhibitor and/or an EGFR inhibitor). Accordingly, in context of the present invention, the herein described method of treatment may also comprise the co-administration of additional compounds/medicaments and the herein defined (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib). In particular, the method of treatment preferably comprises the co-administration and the herein defined (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib). These co-therapy approaches and combination therapy approaches are envisaged with respect to the present invention.

The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of partially or completely curing a disease and/or adverse effect attributed to the disease. The term "treatment" as used herein covers any treatment of a disease in a subject and includes: (a) preventing and/or ameliorating a cancerous disease from occurring in a subject which may be predisposed to the disease; (b) inhibiting the disease, i.e. arresting its

development like the inhibition of cancer progression and/or inhibition of the development of metastases; or (c) relieving the disease, i.e. causing regression of the disease, like the repression of a tumour and/or of metastases.

In accordance with the present invention, the term "prevention" or "preventing" of an cancer/cancerous disease means the cancer per se can be hindered of developing or to develop into an even worse situation. Accordingly, it is one of the advantages of the present invention that an (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) can be employed in avoidance of cancer, cancer progression, and/or the development of metastases. Therefore, in accordance with the present invention, an (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) may also be employed before a cancer develops.

However, as disclosed and provided herein, an (*S*)-enantiomer of an aminoheteroaryl compound (such as the (*S*)-enantiomer of crizotinib) may also be employed in the amelioration and/or treatment of disorders wherein the diseased status has already developed, i.e. in the treatment of an existing cancer. Accordingly, the term "treatment" as used herein also relates to medical intervention of an already manifested disorder, like the treatment of an already defined and manifested cancer.

The terms "patient" and "subject" are used interchangeably herein. A "patient" or "subject" for the purposes of the present invention may be a vertebrate. Said vertebrate may be a mammal, such as a human. Said vertebrate may also be farm animal, such as a cow, pig, sheep, goat, horse, camel, chicken, turkey or other commercially important farm animals. A "patient" or "subject" for the purposes of the present invention includes both humans and other animals, particularly mammals, and other organisms. Thus, the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

The "patient" or "subject" to be treated or in need of treatment according to this invention may be a vertebrate. Said vertebrate may be a mammal, such as a human. Said vertebrate may also be farm animal, such as a cow, pig, sheep, goat, horse, camel, chicken, turkey or other commercially important farm animals. The "patient" or "subject" to be treated or in need of treatment according to this invention is preferably a mammal. The "patient" or "subject" to be treated is most preferably a human. In particular, the "patient" or "subject" to be treated is a human patient that suffers from cancer.

In accordance with the present invention, the term “status”, in particular “NUDT1/MTH1-status”, “RAS-status”, “ALK-status” or “c-Met-status” relates to the genetic constitution of the gene of the respective protein, the expression of the respective protein and/or the biological activity of the respective protein. In particular, the “status” of NUDT1/MTH1, RAS, ALK or c-Met may be reflected by the presence or absence of activating or inactivating aberrations of NUDT1/MTH1, RAS, ALK or c-Met, respectively. Accordingly, the term “status” as used herein also relates to the existence of an activating or inactivating mutation within the gene or protein of interest (e.g. within the gene or protein of NUDT1/MTH1, RAS, ALK or c-Met).

In addition, in context of the present invention, the “status” of a gene or protein also relates to the “level” of the respective gene and/or protein, i.e. the level of NUDT1/MTH1, RAS, ALK, or c-Met. Accordingly, the term “status” in context of, e.g., “NUDT1/MTH1-status” as used herein is known in the art and relates to the level of MTH1 biological activity and/or MTH1 expression (e.g. the level of MTH1 mRNA and/or MTH1 protein). Accordingly, the term “RAS-status” as used herein is known in the art and relates to the level of RAS biological activity and/or RAS expression (e.g. the level of RAS mRNA and/or RAS protein). Accordingly, the term “ALK-status” as used herein is known in the art and relates to the level of ALK biological activity and/or ALK expression (e.g. the level of ALK mRNA and/or ALK protein). Accordingly, the term “c-Met-status” as used herein is known in the art and relates to the level of c-Met biological activity and/or c-Met expression (e.g. the level of c-Met mRNA and/or c-Met protein). The level of NUDT1/MTH1, RAS, ALK or c-Met expression may be reflected by the biological activity of NUDT1/MTH1, RAS, ALK or c-Met, respectively. Accordingly, the term “status” as used herein, also relates to the biological activity of a protein, e.g. of MTH1, RAS, ALK or c-Met.

The level of a particular protein (e.g. the expression level and/or the level of the biological activity of said protein) may be increased or decreased. In particular, the level of NUDT1/MTH1, RAS, ALK or c-Met may be increased by activating aberrations of NUDT1/MTH1, RAS, ALK or c-Met, respectively. Alternatively, the level of NUDT1/MTH1, RAS, ALK or c-Met may be decreased by inactivating aberrations of NUDT1/MTH1, RAS, ALK or c-Met, respectively. Accordingly, the term “status” as used herein also relates to the existence of an activating or inactivating mutation within the gene or protein of interest. In addition, in context of the invention, an inactivating aberration may be a mutation resulting in a loss-of function mutant. An inactivating aberration may also be the deletion or partial deletion of a gene encoding the respective protein (e.g. MTH1, RAS, ALK

or c-Met). In line with, in context of the invention, an activating aberration may be a mutation resulting in a protein (e.g. MTH1, RAS, ALK or c-Met) with enhanced expression or activity. Such activating aberration may be, for example, an activating ALK aberration. An activating ALK aberration may be, e.g., a gene mutation or a chromosomal translocation such as EML4-ALK.

In addition, as used herein, the term “patient’s status”, such as “patient’s NUDT1/MTH1-status”, “patient’s RAS-status”, “patient’s ALK-status”, or “patient’s c-Met-status” relates to status of the cancer of said patient, such as the “cancer’s NUDT1/MTH1-status”, the “cancer’s RAS-status”, the “cancer’s ALK-status” or the “cancer’s c-Met-status”. In particular, the term “patient’s status” (such as “patient’s NUDT1/MTH1-status”) relates to the status of the cancer cell or tissue of said patient. Thus, the term “patient’s status” (such as “patient’s NUDT1/MTH1-status”) relates to the status of the cancer cell or tissue of said patient regarding the genetic constitution of the genes of the respective protein, the expression of the respective protein and/or the biological activity of the respective protein.

In this context, the terms “MTH1-positive”, “NUDT1/MTH1-positive”, “positive for MTH1” and “positive for NUDT1/MTH1” are used interchangeably herein and relate to both, *physiological* (i.e. “normal”) and *increased* expression and/or biological activity of NUDT1/MTH1. “Physiological” or “normal” expression and/or biological activity of NUDT1/MTH1 relates to the expression and/or biological activity (i.e. catalytic activity) of MTH1 in cells, which do express MTH1, but which do not have an activating or inactivating MTH1 aberration. “Increased” expression and/or biological activity of NUDT1/MTH1 relates to the expression and/or biological activity (i.e. catalytic activity) of MTH1 in cells, which do express MTH1 and have an activating MTH1 aberration. “Increased” expression and/or biological activity of NUDT1/MTH1 further relates to an increased expression and/or biological activity (i.e. catalytic activity) of MTH1 compared to cells, which do express MTH1, but which do not have an activating MTH1 aberration. An activating MTH1 aberration leads to an increased expression and/or biological activity (i.e. catalytic activity) of MTH1. Accordingly, the term “MTH1-positive” also relates to *increased* expression and/or biological activity of MTH1. Examples for an activating MTH1 aberration are a gene amplification, a gene mutation or a chromosomal translocation leading to an increased expression and/or biological activity (i.e. catalytic activity) of MTH1.

In line with this, the terms “MTH1-negative”, “NUDT1/MTH1-negative”, “negative for MTH1” and “negative for NUDT1/MTH1” are used interchangeably herein and relate

reduced expression and/or biological activity of NUDT1/MTH1. “Reduced” expression and/or biological activity of MTH1 relates to the expression and/or biological activity (i.e. catalytic activity) of MTH1 in cells, which do not express MTH1. “Reduced” expression and/or biological activity of NUDT1/MTH1 further relates to reduced expression and/or biological activity (i.e. catalytic activity) of MTH1 compared to cells, which do express MTH1 and which do not have an inactivating MTH1 aberration. Additionally, “Reduced” expression and/or biological activity of MTH1 relates to the expression and/or biological activity (i.e. catalytic activity) of MTH1 in cells, which do express MTH1, and which have an inactivating MTH1 aberration. Examples for an inactivating MTH1 aberration are a gene mutation or a chromosomal translocation leading to a reduction in expression and/or biological activity (i.e. catalytic activity) of MTH1.

Similarly, as used herein, the terms “c-Met-positive” and “positive for c-Met” are used interchangeably herein and relate to both, *physiological* (i.e. “normal”) and *increased* expression and/or biological activity of c-Met. C-Met-positive cells may be c-Met-dependent, i.e. cells wherein the survival and/or proliferation depends on the expression of c-Met. “Physiological” or “normal” expression and/or biological activity of c-Met relates to the expression and/or biological activity (i.e. catalytic activity) of c-Met in cells, which do express c-Met, but which do not have an activating or inactivating c-Met aberration. “Increased” expression and/or biological activity of c-Met relates to the expression and/or biological activity (i.e. catalytic activity) of c-Met in cells, which do express c-Met and have an activating c-Met aberration. “Increased” expression and/or biological activity of c-Met further relates to an increased expression and/or biological activity (i.e. catalytic activity) of c-Met compared to cells, which do express c-Met, but which do not have an activating c-Met aberration. An activating c-Met aberration leads to an increased expression and/or biological activity (i.e. catalytic activity) of c-Met. Accordingly, the term “c-Met-positive” also relates to an *increased* expression and/or biological activity of c-Met. Examples for an activating c-Met aberration are a gene amplification, a gene mutation or a chromosomal translocation leading to an increased expression and/or biological activity (i.e. catalytic activity) of c-Met.

In line with this, the terms “c-Met-negative” and “negative for c-Met” are used interchangeably herein and relate to *reduced* expression and/or biological activity of c-Met. C-Met-negative cells are c-Met-independent, i.e. cells wherein the survival and/or proliferation does not depend on the expression of c-Met. “Reduced” expression and/or biological activity of c-Met relates to the expression and/or biological activity (i.e. catalytic activity) of c-Met in cells, which do not express c-Met. “Reduced” expression and/or

biological activity of c-Met further relates to a reduced expression and/or biological activity (i.e. catalytic activity) of c-Met compared to cells, which do express MTH1 and which do not have an inactivating c-Met aberration. Additionally, "Reduced" expression and/or biological activity of c-Met relates to the expression and/or biological activity (i.e. catalytic activity) of c-Met in cells, which do express c-Met, and which have an inactivating c-Met aberration. Examples for an inactivating c-Met aberration are a gene mutation or a chromosomal translocation leading to a reduction in expression and/or biological activity (i.e. catalytic activity) of c-Met.

It is noted that the terms "ALK-negative", "negative for ALK" and "do/does not have an activating ALK-aberration" are used interchangeably herein. Accordingly, term "ALK-negative" relates to both, *physiological* (i.e. "normal") and *reduced* expression and/or biological activity of ALK. "Physiological" or "normal" expression and/or biological activity of ALK relates to ALK expression and/or ALK biological activity (i.e. catalytic activity) in cells, which do express ALK, but which do not have an activating ALK aberration. "Physiological" or "normal" expression and/or biological activity of ALK further relates to ALK expression and/or ALK biological activity in a healthy, non-cancerous cell or tissue. This non-cancerous cell or tissue is to be considered as not having an activating ALK-aberration.

"Reduced" expression and/or biological activity of ALK relates, in one aspect, to no expression and/or biological activity (i.e. catalytic activity) of the ALK kinase. "Reduced" expression and/or biological activity of ALK further relates to qualitatively or quantitatively reduced expression and/or biological activity (i.e. catalytic activity) of ALK as compared to physiological ALK expression and/or physiological ALK biological activity as defined herein above.

The terms "ALK-positive", "positive for ALK" and "do/does have an activating ALK-aberration" are used interchangeably herein and relate to a cancer cell or tissue that has an activating ALK aberration. ALK-positive cells may be ALK-dependent, i.e. (cancer and/or tumour) cells wherein the survival and/or proliferation depends on the expression of ALK. An activating ALK aberration leads to an increased expression and/or biological activity (i.e. catalytic activity) of ALK. "Increased" expression and/or biological activity of ALK relates to an increased expression and/or biological activity (i.e. catalytic activity) of ALK as compared to cells, which do express ALK, but which do not have an activating ALK aberration. In addition, "increased" ALK expression and/or ALK biological activity relates to an enhanced expression and/or biological activity of ALK as compared to physiological expression and/or

biological activity of the ALK kinase in healthy, non-cancerous cells. Accordingly, the term “ALK-positive” relates to *enhanced* expression and/or biological activity of ALK. Examples for an activating ALK aberration are a gene amplification, a gene mutation or a chromosomal translocation leading to an increased expression and/or biological activity (i.e. catalytic activity) of ALK. An example for a chromosomal translocation which represents an activating ALK aberration is EML4-ALK.

The gene name of the protein MTH1 is NUDT1. At present, four isoforms of NUDT1/MTH1 (p18, p21, p22 and p26) have been reported of which p18 is considered the dominant isoform. The isoform p18 has been used in the experiments described herein. Nucleotide and amino acid sequences of NUDT1/MTH1 are provided herein, below. Mutations for MTH1 have been reported, however, their physiological or clinical relevance has not been elucidated. Methods for determining the NUDT/MTH1-status (e.g. for detecting the level (such as the expression level) of NUDT/MTH1 or for determining whether the nucleotide or amino acid sequence of NUDT/MTH1 contains a specific mutation) are known in the art and described herein below. With respect to RAS, several genes exist (i.e. HRAS, KRAS, NRAS, and MRAS). Due to alternative splicing there are several existing isoforms. Known activating RAS mutations include mutations of KRAS, e.g., the G12 mutation (e.g. G12C) or the Q61 mutation (e.g. Q61H). Nucleotide and amino acid sequences of RAS are provided herein, below. In addition, the amino acid sequence for mutant G12C KRAS, which is found, e.g., in lung cancer, is provided herein as SEQ ID No. 23, below. Methods for determining the RAS-status (e.g. for detecting the level (e.g. the expression level) of RAS or determining whether the nucleotide or amino acid sequence of RAS contains a specific mutation) are known in the art and described herein below. In addition, herein disclosed are nucleotide and amino acid sequences of ALK and c-Met (see below). Furthermore, disclosed herein are sequence annotations of the amino acid sequences of RAS, ALK and c-Met, providing several aberration and mutations of RAS, ALK and c-Met, respectively (see below).

Activating and inactivating aberrations include genetic aberrations such as gene mutation, gene copy number increase, aberration of gene expression, and aberration of mRNA expression. A general overview of genetic aberration techniques for detecting said aberrations is shown in Table 1, below.

Tabelle 1: General overview of genetic aberrations and techniques for detecting genetic aberrations.

Aberration	Test material	Location	Method of detection
Gene mutation	DNA	nucleus	Mutation analysis such as sequencing etc.
Gene copy number increase	DNA	nucleus	Fluorescent In-Situ Hybridisation (FISH)
Gene expression	Protein	depending on gene	Immunohistochemistry (IHC)
Gene mRNA expression	RNA	nucleus	RT-PCR

Other techniques that can be used to detect NUDT1/MTH1, ALK, RAS or c-Met aberrations (e.g. genetic aberrations), such as amplifications or mutations in DNA derived from tumour biopsies, include Peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, PCR-Invader, SNaPshot, PCR/HRMA/dHPLC, PCR/firFLP, Fluorescent In-Situ Hybridisation (FISH) and Immunohistochemistry (IHC) as described herein, below.

Peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp is a rapid and sensitive detection system for mutations that can detect, for instance, EGFR mutations in the presence of 100–1000-fold background of wild-type EGFR from non-tumour cells.²⁴ This method is known in the art and described, e.g., in Nagai (2005) Cancer Res 65: 7276-7282.²⁴

PCR-Invader. This system uses two simultaneous reactions to identify known single nucleic acid changes in DNA sequence and to amplify the signal. This method is commonly known in the art and described, e.g., in Tadokoro (2011) Transl Res. 158:169-79 as well as on <http://www.twt.com/invader/invader.html>.

SNaPshot. SNaPshot is a single nucleotide primer extension assay that can be used to detect known single nucleotide mutations. This method is commonly known in the art and described, e.g., in Hurst (2009) BMC Research Notes 2:66 as well as on <https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=600762&tab=DetailInfo>.

PCR/HRMA/dHPLC. Biopsy DNA is amplified by PCR and subjected to high resolution melting analysis and/or denaturing high performance liquid chromatography to detect mutations.^{25,26} This method is commonly known in the art and described, e.g., in Jänne (2006) Clin Cancer Res 12: 751-758²⁵ and in Nomoto (2006) Am J Clin Pathol 126: 608-615.²⁶

PCR/firFLP. DNA is amplified using PCR with fluorescently labelled primers. The fragment is digested with restriction enzymes targeting the region containing the mutation, and the product undergoes fragment analysis to detect digested and undigested fragments.²⁷ This method is commonly known in the art and described, e.g., in Pao (2005) PLoS Med 2 (3): e73.²⁷

Fluorescent In-Situ Hybridisation (FISH) and Immunohistochemistry (IHC). FISH measures the number of copies of DNA of the gene of interest and IHC measures the expression of the according protein within the cell. FISH is commonly known in the art and described, e.g., in Koivunen (2008) Clinical Cancer Research 14: 4275-4283. IHC is also commonly known in the art and described in, e.g., in Chen (2010) Cancer Research 70: 9827-9836. For ALK there is a specific FISH kit available (Abbott Laboratories' Vysis ALK Break Apart FISH Probe Kit (Product Name: Vysis ALK Break Apart FISH Probe Kit, Abbott Order Number: 06N38-020).

For the detection of cytogenetic aberrations several detection methods are known in the art and reviewed, e.g., in Speicher (2005) Nat Rev Genet 6 (10): 782-792.²⁹

All Mutation detection methods should be performed in best practice. This means that all mutation detection methods should be robust and performed to the highest standards with established Standard Operating Procedures (SOPs). Quality control of each step in the process should be in place. How to perform mutation detection methods in best practice is known in the art and described, e.g., in Eberhard (2008) Clin Oncol 26 (6): 983-993.²⁸

Antibodies, probes and primers which may be used for detecting a patient's NUDT1/MTH1-status are commonly known in the art and also shown in the following:

An antibody for detecting a patient's NUDT1/MTH1-status is, e.g., Novus Biologicals MTH1 Antibody (NB100-109).

Primers for detecting a patient's NUDT1/MTH1-status are shown, e.g. in Kennedy (1998) FEBS Lett. 429 (1): 17-20,³⁶ which is herein incorporated by reference in its entirety. In

addition, primers for detecting a patient's NUDT1/MTH1-status are also provided herein, below and in the appended Sequence Listing.

Primer Sequence for the Detection of NUDT1/MTH1 (SEQ ID No. 17)

5P-AGCCTCAGCGAGTTCTCCTG-3P

Primer Sequence for the Detection of NUDT1/MTH1 (SEQ ID No. 18)

5P-GATCTGGCCACCTTGTGC-3P

Antibodies, probes and primers which may be used for detecting a patient's RAS-status are commonly known in the art and also shown in the following:

An antibody for detecting a patient's RAS-status is, e.g., Cell Signaling Ras Antibody #3965.

Primers for detecting a patient's RAS-status are shown, e.g. in Keohavong (1996) Clin. Cancer. Res. 2 (2): 411-418³³; and Gerry (1999) Mol. Biol. 292 (2): 251-262,³⁴ which are herein incorporated by reference in their entirety.

Antibodies, probes and primers which may be used for detecting a patient's c-Met-status are commonly known in the art and also shown in the following:

An antibody for detecting a patient's c-Met-status is, e.g., Cell Signaling Met Antibody #4560

Primers and probes for detecting a patient's c-Met-status are shown, e.g. in Kubo (2009) Int. J. Cancer 124 (8): 1778-1784,³⁵ which is herein incorporated by reference in its entirety.

Antibodies, probes and primers which may be used for detecting a patient's ALK-status are commonly known in the art and also shown in the following:

Antibodies for detecting a patient's ALK-status are, e.g., Cell Signaling #3633 ALK (D5F3) XP® Rabbit mAb, and ALK (C26G7) Rabbit mAb #3333.

Probes and primers for detecting a patient's ALK-status are shown, e.g. in Koivunen (2008) Clin. Cancer. Res. 14 (13): 4275-4283³⁰; and Lin (2009) Mol. Cancer Res. 7 (9): 1466-1476,³¹ which are herein incorporated by reference in their entirety.

Further primers for detecting a patient's ALK-status are shown, e.g., in Chen (2008) Nature 455 (7215): 971-974,³² which is herein incorporated by reference in their entirety.

Antibodies, probes and primers which may be used for detecting a patient's p21-status are commonly known in the art and also shown in the following:

Polynucleotides capable of detecting p21 (e.g. by using qPCR) are the human p21/WAF1-oligonucleotides which have the following sequence:

5'-CTGTGATGCGCTAATGGCG-3' (SEQ ID No. 49)

5'-AAGTCGAAGTTCCATCGCTCA-3' (SEQ ID No. 50)

(See, e.g., Zeng (2006) Cancer Research 66, 10701-10708)

Antibodies capable of detecting p21 are, e.g., the anti-p21 antibody (ab7960), Abcam, and the p21 Waf1/Cip1 (DCS60) Mouse mAb #2946, Cell Signaling.

It is noted that the term "polynucleotide(s)" as used herein encompasses primer(s) and probe(s). Accordingly, the term "polynucleotide(s)" as used herein means both, primer(s) and probe(s). Primers (e.g. primers for detecting a patient's NUDT1/MTH1-status, a patient's RAS-status, a patient's ALK-status and/or a patient's c-Met-status) can, e.g., be designed using available software, such as Primer3Plus (available the following URL: bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; see Rozen and Skaletsky, In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, N.J., pp. 365-386, 2000) and Invitrogen Vector NTI proprietary software. Other methods for designing primers are known to those of skill in the art.

The following figures show and illustrate the present invention:

Figure 1: Chemical proteomics workflow (The Figure is a modified version of a Figure taken from Superti-Furga (2012) *Designing Multi-target Drugs*, (eds. Richard Morphy and John Harris), Drug Discovery Series, Royal Society of Chemistry, Cambridge⁸, which is herein incorporated by reference in its entirety).

Figure 2: Structure of crizotinib and the newly synthesised coupleable derivatives.

Figure 3: Chemical structures of both crizotinib enantiomers.

Figure 4: Evaluation of immobilized compounds with respect to their ability to bind ALK. Only CeMM-146 effectively enriches for both ALK isoforms expressed in SH-SY5Y cells.

Figure 5: Identified interactors of crizotinib (labels are official gene symbols). Kinases are depicted as circles, non-kinase proteins are shown as diamonds.

Figure 6: MTH1 inhibition assay. Two different batches of racemic crizotinib are approx. 10-fold more potent than optically pure (R)-crizotinib in inhibiting MTH1 catalytic activity. In contrast, the promiscuous BCR-Abl kinase inhibitor bosutinib does not exhibit any significant MTH1 inhibition.

Figure 7: MTH1 inhibition assay. The N-aminopropyl-substituted crizotinib derivative CeMM-146 which was prepared from racemic crizotinib also exhibits nanomolar inhibition of MTH1 catalytic activity and is more potent than optically pure (R)-crizotinib.

Figure 8: MTH1 inhibition assay. The N-aminohexylcarboxylic acid derivative CeMM-144 prepared from racemic crizotinib inhibits MTH1 catalytic activity with an IC₅₀ value of approximately 500nM.

Figure 9: MTH1 inhibition assay. Introduction of a PEG-based aminoalkyl substituent at the piperidine ring of racemic crizotinib does not interfere with MTH1 inhibitory activity.

Figure 10: Western blot analysis of SK-ES-1 Ewing's sarcoma cell lysates showing differential MTH1 binding potencies for optically pure (R)-crizotinib versus the racemate.

Figure 11: MTH1 inhibition assay. The (*S*)-enantiomer of crizotinib is a low nanomolar inhibitor of MTH1 catalytic activity. The (*S*)-enantiomer of crizotinib gives a significantly lower IC₅₀ value when compared to the (*R*)-enantiomer or the racemate, indicating that the (*S*)-configuration leads to an increased affinity of the compound. Results indicate technical replicates $\pm$ SEM representative for at least duplicate experiments ($n \geq 2$).

Figure 12: Colony formation assay. MCF 7 (top) and MDA-MB-231 (bottom) human breast cancer cells were seeded in 6-well format followed by drug or mock treatment at the indicated concentrations 24 hours after plating. After four weeks, cells were fixed and stained with crystal violet.

Figure 13: Preparation of CeMM-144, CeMM-145, and CeMM-146.

Figure 14: Stereoselective preparation of the (*S*)-enantiomer of crizotinib. Reagents and conditions: (a) Ph₃P, DIAD, THF, 0 °C, 4 h; (b) Fe, AcOH/EtOH, reflux, 1 h; (c) NBS, MeCN, 0 °C, 15 min; (d) (Boc)₂O, DMAP, DMF, ambient temperature, 18 h; (e) Pd(dppf)₂Cl₂, KOAc, DMSO, 80 °C, 18 h; (f) Pd(Ph₃P)₂Cl₂, Na₂CO₃, DME/H₂O, 87 °C, 16 h; (g) deprotection (e.g. 4 N HCl, 1,4-dioxane/CH₂Cl₂, 40 °C, 12 h).

Figure 15: Cocrystal structure of the (*S*)-enantiomer of crizotinib bound to human MTH1.

Figure 16: MTH1 inhibitor pharmacophore showing preferred structural features of aminoheteroaryl-based MTH1 inhibitors.

Figure 17: A) Comparison of antiproliferative efficacy of the (*S*)-enantiomer of crizotinib versus SCH51344 against SW480 cells. B) ITC for MTH1 with (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib. C) The (*S*)-enantiomer of crizotinib inhibits colony formation of PANC1 (pancreatic cancer) and SW480 (colon carcinoma) cells.

Figure 18: A) Comet assay. Both SCH51344 and the (*S*)-enantiomer of crizotinib, but not (*R*)-crizotinib, induce single strand breaks (positive control = H₂O₂ (150 μ M, 10 min; MTM,

mean tail moment.), and B) increase 53BP1 foci in SW480 cells. C) Cocrystal structure of the (*S*)-enantiomer of crizotinib bound to human MTH1. Hydrogen-bonding interactions are shown by dashed lines. D) MTH1 interactions with (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib. Left panel shows (*R*)-crizotinib; the thinner lines indicate part of the (*R*)-crizotinib that was not resolved in the electron density. Right panel shows the (*S*)-enantiomer of crizotinib; alternate protein conformations in the absence of the (*S*)-enantiomer of crizotinib are shown in darker colour. E) Results from SW480 mouse xenograft study. Top panel: Effect on tumour growth following 35 days treatment with the MTH1 inhibitor (*S*)-crizotinib (*i.e.* the (*S*)-enantiomer of crizotinib) (25mg/kg qd, sc daily) (Survival curves significant different, $p < 0.01$ (Mantel-Cox test)). Bottom panel: Effect on tumour growth on termination day (35 days treatment) (data shown as mean $\pm$ SEM).

Figure 19: The (*S*)-enantiomer of crizotinib does not exhibit any increased unspecific cytotoxicity compared to (*R*)-crizotinib. In contrast, the (*R*)-enantiomer significantly impairs the growth of untransformed BJ skin fibroblasts at low micromolar concentrations in a colony formation assay. Compounds were added 24h after seeding the cells and plates were incubated for 10 days, washed, fixed, and stained with crystal violet. Images are representative for duplicate experiments.

Figure 20: Pharmacologic c-Met kinase inhibition by a highly potent inhibitor (JNJ-38877605) does not suppress growth of K-Ras-mutated SW480 cells in contrast to the MTH1 inhibitors SCH51344 and the (*S*)-enantiomer of crizotinib.

Figure 21: A) The MTH1 inhibitors SCH51344 and the (*S*)-enantiomer of crizotinib, but not (*R*)-crizotinib, increase the number of 53BP1 foci in SW480 cells. B) Similarly, transient knock-down of MTH1 also induces formation of 53BP1 foci in SW480 cells.

Figure 22: A) MTH1 crystal structure overview with the (*S*)-enantiomer of crizotinib. B) As A) with a molecular surface shown covering MTH1 apart from the binding site loops.

Figure 23: MTH1 crystal structures with (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib showing $2F_o - F_c$ electron density maps contoured at 1σ . A) (*R*)-Crizotinib B) the (*S*)-enantiomer of crizotinib.

Figure 24: Meta-analysis of Oncomine data. MTH1 expression strongly correlates with upregulated Ras which is also reflected by the fact that cancers with high prevalence of Ras mutations such as lung and colon carcinoma express higher levels of MTH1 than other unrelated cancer types.

Figure 25: Synthesis of compound 2. *tert*-Butyl (2-(2-(2-((6-methoxy-3-methyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-yl)amino)ethoxy)ethoxy)ethyl)carbamate (4). According to literature (US5608067A) sodium hydride (3.3 mg, 0.087 mmol, 60% dispersion) was added to *N*-Boc- 2,2'-(ethylenedioxy)diethylamine (723 mg, 2.8 mmol) and the mixture stirred for 20 min at room temperature. Upon addition of 4-chloro-6-methoxy-3-methyl-1*H*-pyrazolo[3,4-*b*]quinoline (3) (Yang (2012) Bioorganic & Medicinal Chemistry Letters 22, 235-239) (18 mg, 0.07 mmol) the reaction was heated to 110 °C and stirred overnight. After cooling, water (5 mL) was added followed by extraction with ethyl acetate (3×10 mL). The organic layer was dried over sodium sulphate, filtered, and concentrated in vacuo. Flash column chromatography (dichloromethane/ethanol 10:1) gave a yellowish crystalline solid. Yield: 12.2 mg (37%). ¹H NMR (400 MHz, DMSO) δ 12.50 (s, 1H), 7.64 (d, *J* = 9.3 Hz, 1H), 7.55 (d, *J* = 2.7 Hz, 1H), 7.30 (dd, *J* = 9.3, 2.7 Hz, 1H), 6.68 (s, 1H), 6.21 (s, 1H), 3.88 (s, 3H), 3.82 (q, *J* = 5.5 Hz, 2H), 3.67 (t, *J* = 5.5 Hz, 2H), 3.55 – 3.49 (m, 2H), 3.44 (dd, *J* = 5.9, 3.5 Hz, 2H), 3.35 – 3.29 (m, 2H), 3.01 (q, *J* = 11.9, 6.0 Hz, 2H), 2.70 (s, 3H), 1.35 (s, 9H); MS ESI *m/z* (relative intensity, %) 360 [*M*⁺ + H] (100).

N-(2-(2-(2-Aminoethoxy)ethoxy)ethyl)-6-methoxy-3-methyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amine (2). Trifluoroacetic acid (40 μL) was added to a solution of compound 4 (8 mg, y mmol) in dichloromethane (4 mL) and the mixture was stirred at room temperature for 45 min. After removal of solvents, the crude product was purified by MPLC (dichloromethane/methanol 9:1) to give a yellow wax-like solid. Yield: 3 mg (48%). ¹H NMR (400 MHz, DMSO) δ 12.53 (s, 1H), 7.65 (d, *J* = 9.3 Hz, 1H), 7.57 (d, *J* = 2.7 Hz, 1H), 7.31 (dd, *J* = 9.3, 2.7 Hz, 1H), 6.25 (s, 2H), 3.89 (s, 3H), 3.83 (t, *J* = 5.6 Hz, 2H), 3.68 (t, *J* = 5.5 Hz, 2H), 3.61 – 3.37 (m, 7H), 2.83 (t, *J* = 5.3 Hz, 2H), 2.70 (s, 3H); MS ESI *m/z* (relative intensity, %) 460 [*M*⁺ + H] (97), 404 (100), 360 (47).

Figure 26: Stable knockdown of MTH1 impairs colony formation of SW480 cells. Stable knockdown of MTH1 significantly reduced colony formation of SW480 cells.

Figure 27: Derivatisation of (*S*)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)-5-(1-(piperidin-4-yl)-1H-pyrazol-3-yl)pyridin-2-amine to yield (*S*)-5-(1-(1-(3-aminopropyl)piperidin-4-yl)-1H-pyrazol-3-yl)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-2-amine.

The Figure shows (*S*)-tert-butyl (3-(4-(3-(6-amino-5-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-3-yl)-1H-pyrazol-1-yl)piperidin-1-yl)propyl)carbamate. ¹H NMR (400 MHz, CDCl₃) δ 7.74 (d, *J* = 1.7 Hz, 1H), 7.58 – 7.46 (m, 2H), 7.30 (dd, *J* = 8.9, 4.8 Hz, 1H), 7.04 (dd, *J* = 8.9, 7.9 Hz, 1H), 6.87 (d, *J* = 1.7 Hz, 1H), 6.07 (q, *J* = 6.7 Hz, 1H), 4.78 (s, 2H), 4.18 – 4.02 (m, 1H), 3.48 (s, 1H), 3.20 (d, *J* = 6.0 Hz, 2H), 3.05 (d, *J* = 11.8 Hz, 2H), 2.47 (t, *J* = 6.7 Hz, 2H), 2.27 – 1.95 (m, 6H), 1.85 (d, *J* = 6.7 Hz, 3H), 1.75 – 1.62 (m, 2H), 1.44 (s, 9H); ESI-MS: 607 (*M* + *H*).

The Figure further shows (*S*)-5-(1-(1-(3-aminopropyl)piperidin-4-yl)-1H-pyrazol-3-yl)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-2-amine. ¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, *J* = 1.5 Hz, 1H), 7.61 – 7.46 (m, 2H), 7.30 (dd, *J* = 8.9, 4.8 Hz, 1H), 7.04 (dd, *J* = 8.9, 7.9 Hz, 1H), 6.87 (d, *J* = 1.6 Hz, 1H), 6.07 (q, *J* = 6.7 Hz, 1H), 4.76 (s, 2H), 4.20 – 4.01 (m, 1H), 3.07 (d, *J* = 11.7 Hz, 2H), 2.81 (t, *J* = 6.5 Hz, 2H), 2.47 (t, *J* = 7.1 Hz, 2H), 2.23 – 1.94 (m, 6H), 1.85 (d, *J* = 6.7 Hz, 3H), 1.80 (s, 2H), 1.73 – 1.63 (m, 2H). ESI-MS: 507 (*M* + *H*).

Figure 28: Anti-cancer specificity. Various isogenic BJ fibroblast cell lines were treated with several concentrations of the (*S*)-enantiomer of crizotinib in colony formation assays. As indicated, the investigated cell line panel comprised non-transformed (“wildtype”) cells, cells immortalized with telomerase (hTERT), cells transformed with hTERT and SV40 Large T antigen (SV40T), and cells transformed with hTERT, SV40T, and the KRAS mutant V12.

Figure 29: Inhibition of MTH1-catalytic activity by the (*S*)-enantiomer of crizotinib is not substrate-dependent. The Figure shows that the (*S*)-enantiomer of crizotinib also inhibits hydrolysis 2-OH-dATP by MTH1 in a dose-dependent manner.

Figure 30: Effect of p53-/p21-status and mismatch repair pathways on the activity of the (*S*)-enantiomer of crizotinib. The Figure shows that p53 status and presence or absence of functional MLH1 do not affect the activity of the (*S*)-enantiomer of crizotinib. However, loss of p21 seems to increase the sensitivity of HCT116 cells toward treatment with the (*S*)-enantiomer of crizotinib.

Figure 31: MTH1 enzymatic function increases the content of 8-oxo-guanine in DNA. A) and B) Treatment of BJ fibroblasts with (*S*)-, but not (*R*)-crizotinib, increased staining of an anti-8-oxo-guanine mouse monoclonal antibody. At the same time, staining for 53BP1, a specific marker for DNA damage, is increased, which is in line with the effect observed for anti-MTH1 siRNA.

Figure 32: The (*S*)-enantiomer of crizotinib activates DNA repair mechanisms and induces the formation of single strand breaks due to activated base excision repair (BER). The (*S*)-enantiomer of crizotinib has been investigated in an alkaline comet assay, which reveals DNA single strand breaks by single cell electrophoresis. Using SW480 cells it was found that both (*S*)-crizotinib, but not (*R*)-crizotinib, yields a significant tail moment in the comet assay. Also an increase of ATM phosphorylation was observed indicating DNA damage pathway activation, again demonstrating an (*S*)-crizotinib-specific effect.

The following non-limiting examples illustrate the invention:

Materials and Methods:

General Information. NMR spectra were recorded on a Bruker Avance III 400 (Bruker, Billerica, MA, U.S.). Chemical shifts are given in ppm, and coupling constants are given in hertz. Mass spectra were recorded using a XeVo-UPLC-TQ-MS system (Waters, Milford, MA, U.S.). Purification by flash column chromatography (FCC) was done using silica gel 60 (Merck, Darmstadt, Germany), MPLC was performed on a Biotage Isolera system (Biotage, Uppsala, Sweden). The purity of the synthesized compounds was determined and confirmed by UPLC analysis.

All synthesis chemicals were purchased from Sigma-Aldrich and Santa Cruz and used without further purification.

Chemicals. All chemicals were purchased from Sigma Aldrich or Fluka (Sigma Aldrich Austria) and used without further purification. Racemic (RS)-crizotinib and JNJ-38877605 were obtained from Selleck Chemicals (Selleck Chemicals LLC, Houston, TX, USA), (*R*)-crizotinib was purchased from Selleck Chemicals, Tocris (Tocris Bioscience, Bristol, UK), and ChemieTek (ChemieTek, Indianapolis, IN, USA). The (*S*)-enantiomer of crizotinib was obtained from ChemFuture (ChemFuture PharmaTech Ltd, Jiangsu, China). SCH51344 was

purchased from Calbiochem (EMD Millipore, Billerica, MA, USA). Synthesis of compound 2 is shown in Figure 25.

Immobilization and affinity purification. Drug-affinity matrices were prepared essentially as described previously (Rix (2007) *Blood* 110, 4055-4063). Briefly, 25 nmol of compound was immobilised on 50 μ L NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Affinity chromatography and elution were performed in duplicate as reported previously, (Fernbach (2009) *Journal of Proteome Research* 8, 4753-4765) using 10 mg total cell lysate as protein input per replicate.

Solution tryptic digestion and peptide purification. After elution, enriched proteins were reduced with dithiothreitol, cysteine residues alkylated by incubation with iodoacetamide and the samples digested with modified porcine trypsin (Promega, Madison, WI). Three percent (and multiples thereof) of the digested eluates were purified and concentrated by C18 reversed-phase material for subsequent duplicate analysis by gel-free one-dimensional liquid chromatography mass spectrometry (1D-LCMS). Details of the LCMS methodology are as previously described (Maurer (2012) *Journal of Proteome Research* 12, 1040-1048).

Protein identification. Peak extraction and conversion of RAW files into the MGF format for subsequent protein identification was performed with msconvert (ProteoWizard Library v2.1.2708). An initial database search was performed with broader mass tolerance to re-calibrate the mass lists for optimal final protein identification. For the initial protein database search, Mascot (www.matrixscience.com, version 2.3.02) was used. Error tolerances on the precursor and fragment ions were ± 10 ppm and ± 0.6 Da, respectively, and the database search limited to fully-tryptic peptides with maximum 1 missed cleavage, carbamidomethyl cysteine and methionine oxidation set as fixed and variable modifications, respectively. The Mascot peptide ion score threshold was set to 30, and at least 3 peptide identifications per protein were required. Searches were performed against the human UniProtKB/SwissProt database (www.uniprot.org release 2012-05) including all protein isoforms.

The initial peptide identifications were used to deduce independent linear transformations for precursor and fragment masses that would minimize the mean square deviation of measured masses from theoretical. Re-calibrated mass list files were searched against the same human protein database by a combination of Mascot and Phenyx (GeneBio, SA, version 2.5.14) search engines using narrower mass tolerances (± 4 ppm and ± 0.3 Da). One missed tryptic

cleavage site was allowed. Carbamidomethyl cysteine was set as a fixed modification and oxidized methionine was set as a variable modification. To validate the proteins, Mascot and Phenyx output files were processed by internally-developed parsers. Proteins with ≥ 2 unique peptides above a score T_1 , or with a single peptide above a score T_2 were selected as unambiguous identifications. Additional peptides for these validated proteins with score $> T_3$ were also accepted. For Mascot searches, the following thresholds were used: $T_1=14$, $T_2=40$ and $T_3=10$; Phenyx thresholds were set to 4.2, 4.75 and 3.5, respectively ($P\text{-value} < 10^{-3}$). The validated proteins retrieved by the two algorithms were merged, any spectral conflicts discarded and grouped according to shared peptides. A false discovery rate (FDR) of $<1\%$ for protein identifications and $<0.1\%$ for peptides (including the ones exported with lower scores) was determined by applying the same procedure against a database of reversed protein sequences.

Bioinformatic analysis. Non-specific binders were filtered from the drug pull-downs using the SAINT software (version 2.3.4) (Choi (2011) Nat Meth 8, 70-73). Using protein spectral counts as a measure of protein abundance and comparing the data of a real pull-down versus the negative control experiments, SAINT calculates the probability of a prey protein to be a real bait interactor.

The SAINT probability cut-off threshold was set to 0.99 as TUBA1B and RPS3A, known contaminants observed in more than thousand MS experiments at our institution, had probability of 0.9993 and 0.9882, respectively.

Expression and purification of MTH1 for crystallization. The expression construct was transformed into *E. coli* BL21 (DE3) competent cells containing the pRARE2 plasmid from commercial Rosetta cells. Colonies from the transformation were used to inoculate 100 mL of LB media containing 34 $\mu\text{g/ml}$ chloramphenicol and 50 $\mu\text{g/ml}$ kanamycin. The culture was grown overnight in a baffled shaker flask at 37 °C with shaking. This culture was used to inoculate LB media by adding 10 ml of culture to 1L of LB (containing 50 $\mu\text{g/ml}$ kanamycin) in baffled shaker flasks. When the culture had an OD600 of approximately 0.6 the temperature was reduced to 18 °C and protein expression was induced by addition of isopropyl β -D-1-thiogalactopyranoside to 0.5 mM. The culture was left shaking at 18 °C overnight before the cell pellets were harvested by centrifugation. The cells were resuspended in Binding Buffer (20 mM Imidazole, 500 mM NaCl, 50 mM Hepes pH 7.4, 5% Glycerol)

with the addition of 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.2 mM phenylmethanesulphonyl fluoride (PMSF). The resuspended cells were stored at -20 °C.

The resuspended cells were thawed and lysed by sonication. Polyethyleneimine was added to a concentration of 0.15% and the lysate was centrifuged at 4 °C to remove insoluble material. The supernatant was loaded onto 7.5 ml of nickel-chelating resin. The resin was washed with Binding Buffer, and Binding Buffer containing 40 mM imidazole and then 60 mM imidazole. The protein was eluted with Binding Buffer containing 250 mM imidazole. The hexahistidine tag was removed by overnight treatment with TEV protease at 4 °C. The digested sample was concentrated to 5 ml volume and loaded onto a Superdex200 gel filtration column (HiLoad 16/60, GE Healthcare) pre-equilibrated in GF Buffer (50 mM Hepes pH 7.5, 300 mM NaCl, 0.5 mM TCEP). Fractions containing MTH1 were pooled and passed through a column of 2.5 ml nickel-chelating resin. The flow-through and an elution with GF Buffer containing 10 mM imidazole were combined. The protein identity was verified by electrospray ionization time-of-flight mass spectrometry (Agilent LC/MSD).

The MTH1 complexes were prepared by adding (*R*)-crizotinib or the (*S*)-enantiomer of crizotinib to dilute protein solution at an approximate molar ratio of 10:1. The MTH1:crizotinib complexes were concentrated together by ultrafiltration to a protein concentration of 20 mg/ml.

Crystallization and data collection. MTH1 complexes were crystallised by the sitting drop vapour diffusion method using 150 nL drops as detailed in Table 3. All crystals were cryo-protected in reservoir solution with the addition of 25% (v/v) ethylene glycol and flash-frozen in liquid nitrogen. X-ray diffraction data was collected at 100 K at the DIAMOND synchrotron.

Structure determination and refinement. The diffraction images were processed using MOSFLM (Leslie in *Evolving Methods for Macromolecular Crystallography* Vol. 245 NATO Science Series; eds RandyJ Read & JoelL Sussman; Ch. 4, 41-51; Springer Netherlands, 2007). The integrated data were scaled and merged using AIMLESS (Evans (2006) *Acta Crystallographica* Section D 62, 72-82) and the CCP4 suite of programs (Winn (2011) *Acta Crystallographica* Section D 67, 235-242). The structures were solved by molecular replacement using PHASER (McCoy (2007) *J. Appl. Crystallogr.* 40, 658-674). All structural models were built using COOT (Emsley (2010) *Acta Crystallographica* Section D 66, 486-501) and refined using REFMAC5 (Murshudov (2011) *Acta Crystallographica* Section D 67,

355-367). Ligand restraints were generated with PRODRG (Schüttelkopf (2004) Acta Crystallographica Section D 60, 1355-1363). Molprobit (Chen (2010) Acta Crystallographica Section D 66, 12-21) was used for structure validation. Data collection and refinement statistics can be seen in Table 4.

Cell culture and Immunoblotting. BJ cells were obtained from ATCC. SW480 and DLD1 were kindly provided by Walter Berger, PANC1 were a generous gift from Rudolf Oehler. All other cell lines were obtained from the American Type Culture Collection (ATCC) or Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). SW480, PANC1 and BJ cells were cultivated in DMEM, DLD1 in RPMI. All media contained 10% fetal bovine serum (FBS) and 10 U/mL penicillin/streptomycin (Gibco). MCF-7 were cultivated in DMEM containing 10% FBS, 10 U/mL penicillin/streptomycin, and 0.01 mg/ml bovine insulin. MDA-MB-231 were grown in Leibovitz's L-15 containing 10% FBS and 10 U/mL penicillin/streptomycin. Antibodies. Anti-ALK antibody was obtained from Cell Signaling Technology, anti-MTH1 was purchased from Novus (Novus Biologicals, Littleton, CO, USA). The following antibodies were used according to manufacturer's instructions: rabbit anti-MTH1 (NB100-109, Novus Biologicals), rabbit anti-actin (AAN01, Cytoskeleton), rabbit anti-ALK (C26G7, Cell Signaling Technology, and 513900, Invitrogen, Life Technologies).

Expression of recombinant MTH1. Codon-optimised human MTH1 cDNA subcloned into a pETM-11 vector (Gunther Stier, EMBL) featuring a His-tag and TEV site was obtained from GenScript (GenScript, NJ, USA) and expressed in the *E. coli* strain BL21 DE3 (Life Technologies). After harvesting, bacteria were lysed using buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% glycerol, 5 mM β -mercaptoethanol, 1 mM PMSF) containing lysozyme (Sigma-Aldrich) and DNase I (Roche). His-tagged protein was purified with NiNTA agarose (Qiagen), washed with buffer, and eluted with an imidazole gradient. Following removal of the His-tag by incubation with TEV protease, fractions were dialysed and purified using size-exclusion chromatography (Sephadex, GE Healthcare). Protein concentration of the purified fractions was determined by UV (A_{280}). The identity of the protein was confirmed by MALDI-TOF and protein activity determined by K_m measurement which gave values in accordance with literature data (Svensson (2011) FEBS Letters 585, 2617-2621).

Chemical proteomics experiments. Drug pull-down experiments were essentially carried out as described previously using 10mg total lysate protein per replicate. For competition experiments, the lysate was preincubated with unmodified, genuine crizotinib (final concentration 20 μ M) for 30 minutes. Pull-down eluates were digested with trypsin, labeled with iTRAQ reagent, fractionated by LC, and analysed on an Orbitrap Velos mass spectrometer. Peak list information was extracted from the acquired MS data and searched against the human SwissProt database version v2010.09_20100812 (including isoforms as obtained from varsplic.pl) with the search engines MASCOT (v2.3.02, MatrixScience, London, UK) and Phenix (v2.5.14, GeneBio, Geneva, Switzerland).²⁰ Details of the protein database search criteria are given elsewhere.²¹ Analysis of quantitative proteomics data was performed with the R/Bioconductor package isobar.²²

MTH1 assay. cDNA encoding human MTH1 (NUDT1) and optimized for expression in *E. coli* was purchased from Genscript. His-tagged MTH1 was expressed from pETM-11 in *E. coli* BL-21(DE3) (Stratagene). The bacteria were harvested, lysed and His tagged MTH1 was purified using Ni-NTA agarose (Qiagen). The His-tag was removed by TEV cleavage and the MTH1 protein further purified using anion exchange chromatography at pH 7.5 using a Sephadex column (GE Healthcare). The identity and purity of the protein was confirmed using mass spectrometry. The activity of MTH1 was monitored using 8-oxo-dGTP (TriLink Biotechnologies) using the PPiLight Inorganic Pyrophosphate Assay (Lonza Rockland Inc.). IC₅₀ values were determined using non-linear regression analysis utilizing GraphPad Prism Software.

More specifically, the MTH1 catalytic assay was performed as follows. Half-maximal inhibitory concentrations (IC₅₀) were determined using a luminescence-based assay as described previously (Svensson (2011) FEBS Letters 585, 2617-2621) with some minor modifications. Briefly, serial dilutions of compounds were dissolved in assay buffer (100 mM Tris-acetate pH 8.0, 40 mM NaCl and 10 mM Mg(OAc)₂ containing 0.005% Tween-20 and 2 mM dithiothreitol (DTT). Upon addition of MTH1 recombinant protein (final concentration 2 nM), plates were incubated on a plate shaker for 15 min at room temperature. After addition of 8-oxo-dGTP (TriLink Biotechnologies, final concentration 16 μ M) the generation of pyrophosphate (PPi) as a result of 8-oxo-dGTP hydrolysis by MTH1 was monitored over a time course of 15 min using the PPiLight Inorganic Pyrophosphate Assay kit (Lonza Rockland). IC₅₀ values were determined by fitting a dose response curve to the data points using non-linear regression analysis utilizing the GraphPad Prism software.

siRNA experiments. Both a commercial anti-MTH1 siRNA set (SMARTpool ON-TARGETplus, Dharmacon) as well as a custom-synthesised siRNA (Sigma-Aldrich) were obtained. The custom siRNA sequence was CGACGACAGCUACUGGUUU, AllStars Negative Control siRNA (Qiagen) was used as control. For transfections, cells were seeded in 24-well plates at approximately 30% confluency 24 h prior to siRNA treatment. The next day, medium was aspirated and transfections were performed with INTERFERin (Polyplus) according to manufacturer's instructions using a final siRNA concentration of 10 nM. Cells were incubated for 2-3 days, washed, detached with trypsin and replated in 6-well plates. After 7-10 days, medium was aspirated, cells were washed with PBS, fixed with ice-cold methanol, stained with crystal violet solution (0.5% in 25% methanol) and left to dry overnight. For quantification of results, UV absorbance of crystal violet was determined at 595 nm after solubilisation by 70% ethanol. Data were analysed using the GraphPad Prism software (t test, $P < 0.05$).

Colony formation assay using SW480 cells. One day before treatment, 10^4 cells were seeded per well in 6-well plates and incubated for 24 h. The next day DMSO (equal to highest amount of compound dilution, maximum 0.2%) or compounds in increasing concentrations were added and cells incubated at 37 °C, 5% CO₂, for 7-10 days. After washing with PBS (Gibco), cells were fixed with ice-cold methanol, stained with crystal violet solution (0.5% in 25% methanol) and left to dry overnight. For quantification of results, UV absorbance of crystal violet was determined at 595 nm after solubilisation by 70% ethanol. Data were analysed using non-linear regression analysis utilizing the GraphPad Prism software. (This colony formation assay has also been performed by using MCF 7 and MDA-MB-231 cells leading to comparable results as shown in Figure 12.)

Colony formation assay using MCF 7 and MDA-MB-231 cells. 1000 cells per well were seeded in 2mL complete medium (day 0). Drugs or DMSO (mock) were added 24h later (day 1) with renewal of medium and drugs on day 15 and day 21. Four weeks after plating cells were fixed with methanol followed by staining with crystal violet.

Comet assay. Cells were treated with compounds for 6 days, upon which DNA single-strand breaks were assayed using the comet assay under alkali conditions. For the H₂O₂ control, cells were treated with H₂O₂ (Sigma-Aldrich) in PBS at 150µM for 10 minutes. Cells were washed

twice with PBS, harvested using a rubber scraper, pelleted by centrifugation, resuspended in PBS and mixed with 1% low-gelling-temperature agarose (Sigma type VII) that was maintained at 37°C. The mixture of cells and agarose was layered onto frosted glass slides pre-coated with 0.5% agarose and slides were placed on ice to gel. Slides were maintained in the dark for all subsequent steps. Slides were immersed in pre-chilled lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl pH 7.70, 1% Triton X-100, 1% DMSO) for 1 hour, washed in pre-chilled distilled water 3 times for 20 minutes and incubated for 45 minutes in pre-chilled alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA, 1 % DMSO, pH 12.8). After electrophoresis for 25 minutes at 25 volts, slides were placed at 4°C over-night, in the dark. The following day, slides were neutralized with 0.4 M Tris-HCl pH 7.0 for 1 hour and stained with SYBR Gold (Invitrogen, diluted 1:10,000 in distilled water) for 30 minutes. Comet tail moments (defined as the average distance migrated by the DNA multiplied by the fraction of DNA in the comet tail) were scored using the CellProfiler cell image analysis software.

Indirect immunofluorescence. Cells were treated with compounds for 6 days, following which they were adhered to glass coverslips, washed with PBS and then fixed with 3% paraformaldehyde in PBS for 20 minutes. Fixed cells were rinsed with PBS and permeabilised with 0.5% Triton-X-100 for 5 minutes. PBS washed slides were incubated for 1 hour with 10 % FCS and 0.1% Triton-X-100 in PBS following which cells were stained with an anti-53BP1 monoclonal antibody (H-300, Santa Cruz, diluted 1:600), in 10 % FCS and 0.1% Triton-X-100 in PBS. After rinsing with PBS coverslips were incubated with an Alexa fluor[®] 568 goat anti-rabbit IgG secondary antibody for 1 hour (Invitrogen, diluted 1:400) in 10 % FCS and 0.1% Triton-X-100 in PBS. After a PBS wash, DNA was counterstained with DAPI (Sigma-Aldrich) for 10 minutes and the coverslips were mounted in Fluorescent Mounting Medium (Dako). Images were analysed with a Zeiss fluorescent microscope at 63 times magnification with supporting software.

Xenograft study. SCID mice (female, 5-6 weeks, Scanbur, Germany) were s.c. injected with 1×10^6 SW480 cells together with a matrix gel (1:1) in the sacral area. Treatment was initiated one day after cell inoculation. Vehicle or MTH1 inhibitor was administered subcutaneously once daily at 25mg/kg for 35 days. MTH1 inhibitor was diluted in 1% DMSO, 10% ethanol, 10% cremaphore, 10% Tween 80, 69% PBS. Tumour size was measured twice weekly and body weight once weekly. At termination, a gross postmortem

inspection was performed; blood was collected for haematological parameters and ASAT, ALAT, creatinine measurements and tumours collected in paraformaldehyde (4%) and later paraffin inbedded for immunohistology analysis. All experiments involving animals followed protocols approved by Stockholms Norra djurförsöksetiska nämnd (laboratory animal ethical committee Stockholm). All animals were acclimatised for one week, and had free access to water and food during the experiment. Animals were under a 12-hour lightcycle, and temperature and humidity according to laboratory animal guidelines and regulations.

8-oxo-guanine staining. 5000 BJ cells were seeded per well in a 24-well plate and after 24h treated with compounds or DMSO as indicated. Cells were fixed 72 h later and stained with the respective antibody (anti-oxoguanine 8 antibody [2Q2311] (ab64548), abcam).

Experiments and Results:

Example 1: Immobilization of crizotinib and derivatives of crizotinib and their binding to ALK

To examine crizotinib's cellular interactors a direct chemical proteomics approach has been pursued in which the compound of interest is immobilized on sepharose beads (Figure 1, "Compound-centred chemical proteomics").⁸ The immobilization is achieved by a chemical reaction which establishes a covalent bond between the compound and the bead matrix. Therefore the compound requires an adequate reactive functional group for the reaction to take place which is why in many cases enforces chemical modification of the original compound. Of course, changing the molecular structure can alter the interaction behavior and target spectrum of the compound to be investigated. It should be noted that also the location within the molecule where the compound is immobilized can affect binding. Thus, a common practice is to check and confirm binding of already known targets for all coupleable derivatives. Three derivatives of crizotinib have been prepared with different chemical linkers covering various linker lengths and chemical nature in terms of hydrophobicity (Figure 2).

Crizotinib has a chiral centre at the carbon to which the methyl (CH₃) group is attached. Therefore there are two enantiomers of crizotinib, i.e. (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib (Figure 3). Clinically, only (*R*)-crizotinib is used as this enantiomer has been shown

to be considerably more potent than the (*S*)-enantiomer in inhibiting c-Met kinase activity (c-Met K_i (*R*) = 0.002 μ M, K_i (*S*) = 0.161 μ M).⁹ In fact, crizotinib was actually developed as a specific c-Met kinase inhibitor. To assess the complete interactome considering both enantiomers, racemic crizotinib (a 1:1 mixture of (*R*)- and (*S*)-enantiomer) was used in the drug pull-down-assays.

Chemical synthesis of crizotinib derivatives CeMM-144, CeMM-145, and CeMM-146 is outlined in Figure 13.

As crizotinib itself is also compatible with the immobilization procedure due to the presence of an N-unsubstituted piperidine moiety it was included in the linker evaluation experiment with CeMM-144, CeMM-145 and CeMM-146. To confirm that the immobilization did not interfere with crizotinib's ability to bind its cognate targets, drug-bead matrices were generated for all four compounds followed by pull-down experiments using ALK-positive SH-SY5Y neuroblastoma cells. Bosutinib, a promiscuous BCR-Abl kinase inhibitor which also binds ALK,¹⁰ was used as positive control. The eluates were analysed by SDS-PAGE and Western blot (Figure 4).

As shown in Fig. 4, SH-SY5Y cells express two ALK isoforms with different molecular weight. The aminopropyl-substituted CeMM-146 enriches both ALK isoforms to highest extent, followed by the PEG-derivative CeMM-145. Unmodified crizotinib and CeMM-144 bind ALK to a lesser extent than CeMM-146 and CeMM-145. Based on these results it was decided to use CeMM-146 in all future experiments.

Example 2: The Identification of interactors of crizotinib: MTH1 as a new target of crizotinib

For the profiling, three human cancer cell lines with different genetic background were selected: SH-SY5Y (NB, ALK-positive), NCI-H3122 (NSCLC, EML4-ALK-positive), and NCI-H1648 (c-Met amplification, ALK-negative). To distinguish direct binders from indirect or unspecific binders competition experiments were conducted in which the cell lysates were preincubated with unmodified crizotinib for 30 minutes at a final concentration of 20 μ M before the affinity purification step. Isobaric tag for relative and absolute quantification (iTRAQ) labeling was used to quantify peptides in MS experiments. All experiments were

carried out in duplicates. Comparing the ratio of labeled peptides in normal versus competitive pull-downs an affinity ranking was generated, prioritizing the most efficiently competed proteins as they are likely to be the top direct interactors of crizotinib. The results of the analyses for the top identified proteins from all three cell lines are summarized in Figure 5.

As expected, ALK could be identified as a target in both the ALK-positive SH-SY5Y and H3122 samples whereas c-Met was captured in H1648 and H3122 pull-downs confirming the overall experimental strategy. Interestingly, apart from several unknown kinase targets the 7,8-dihydro-8-oxoguanine-triphosphatase MTH1 (gene symbol: NUDT1) was discovered as a common target of crizotinib in all samples.

The result that MTH1 interacts with crizotinib has been confirmed by screening a small kinase inhibitor collection in a thermal shift stability assay using recombinant MTH1 protein. By applying this thermal shift stability assay, the inventors discovered that the dual Met/ALK inhibitor crizotinib exhibits high affinity toward MTH1 (data not shown).

Human MutT homologue 1 (MTH1) depicts the major clearance enzyme for oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP.¹¹ Oxidised nucleotides, which are generated by attack of reactive oxygen species (ROS) on DNA or the nucleotide pool, can cause DNA damage and mutations. Sources of ROS include mitochondrial respiration, chemicals or radiation. The frequently occurring 8-oxo-guanine (8-oxo-G), for example, can lead to transversion mutations during replication. In contrast to unoxidised guanine, 8-oxo-G is able to pair with either cytosine or adenine with almost equal efficiency, thereby generating mutations if 8-oxo-G is inserted opposite A in a nascent DNA strand or vice versa. If the misincorporated oxidised nucleotide is recognised by the DNA repair system the lesion can be repaired by base excision repair (BER). BER involves induction of a temporary single strand break to remove the falsely inserted base and subsequent replacement. However, high amounts of 8-oxo-G can lead to accumulation of single strand breaks which eventually progress to double strand breaks (DSB), thus inducing cell cycle arrest (quiescence or senescence) and apoptosis. By converting the oxidised triphosphate nucleotides into the corresponding monophosphates which can no longer be used as substrates by DNA polymerases, MTH1 prevents integration of oxidised bases into DNA and therefore mutations and oxidative DNA damage induced by ROS. Transformation of cells by oncogenes such as mutant RAS which occurs in about 20% of all tumours can also lead to increased production

of ROS.¹² As for normal cells, oxidative damage caused by ROS can force cancer cells into a state of quiescence or senescence (OIS), and eventually apoptosis. To overcome senescence, RAS-transformed cells upregulate MTH1 which protects the cells from oxidative DNA damage. For instance, it has been shown that human skin fibroblasts transfected with HRAS undergo senescence, but this phenotype can be rescued by concomitant overexpression of MTH1.¹² Consequently, reports indicate that MTH1 suppression causes proliferative defects in cancer cells expressing mutant RAS.¹³ As MTH1^{-/-} knockout mice show a very mild phenotype,¹⁴ targeting MTH1 with small molecules may provide a novel and well-tolerated therapeutic option for the difficult to treat RAS mutant cancers.

Example 3: Crizotinib inhibits the catalytic activity of MTH1

Publicly available protein-protein interaction databases were examined to check if MTH1 might be interacting with a kinase target of crizotinib which could lead to “piggy-backing”, i.e. an indirect interaction which could also cause an enrichment of MTH1 peptides in our analysis. However, no such interactions have been reported. This investigation was extended to a different type of cancer, the Ewing’s sarcoma family of tumours (ESFT) as it was found that crizotinib inhibited the growth of these cells at low nanomolar concentrations. Again, MTH1 was identified in all pull-downs interrogating SK-ES-1 and SK-N-MC cells, both of which are thought to be driven by the EWS-FLI1 oncogene. To confirm that crizotinib is indeed inhibiting the catalytic activity of MTH1, an enzymatic assay was performed using recombinant MTH1. In cells, MTH1 hydrolyses oxidised nucleotides such as 2-OH-dATP and 8-oxo-dGTP, yielding the corresponding monophosphate and pyrophosphate (PPi). A luminescence-based assay was performed which monitors the production of PPi generated by MTH1-mediated 8-oxo-dGTP hydrolysis following a protocol which has been used to determine MTH1 enzyme kinetics.¹⁵ IC₅₀ values were determined for crizotinib considering both the clinically applied, optically pure (*R*)-enantiomer as well as the racemic mixture containing both (*R*)- and (*S*)-enantiomer (Figures 6-9). Confirming the pull-down results, both racemic and pure (*R*)-crizotinib inhibited MTH1’s ability to hydrolyse 8-oxo-dGTP. However, the inhibitory potency of the racemate was about 10 times higher than the one observed for the enantiomerically pure (*R*)-crizotinib. These results were validated by analysis of different batches of both pure (*R*)- and racemic crizotinib which had been obtained from distinct vendors. A different ALK inhibitor, NVP-TAE684,¹⁶ was also examined which did not exhibit any significant inhibition of MTH1 catalytic activity at concentrations of 100µM

(data not shown). This suggests that MTH1 inhibition is not a general phenomenon for ALK kinase inhibitors but is a feature of the particular chemotype of crizotinib. However, as kinase inhibitors mimic ATP when binding to the kinase active site it is likely that other kinase inhibitors are also potent MTH1 inhibitors as its natural substrates are derivatives of ATP and GTP.

In addition, competitive pull-down experiments in SK-ES-1 cells were performed using racemic CeMM-147 as bait and increasing concentrations of either optically pure (*R*)-crizotinib or the racemate (Figure 10). As suggested by the IC₅₀ results, only racemic crizotinib effectively prevented binding of MTH1 at preincubation concentrations up to 20 μM.

To confirm the preference of the (*S*)-enantiomer of crizotinib to bind and inhibit MTH1, we resynthesised an optically pure batch of the (*S*)-enantiomer of crizotinib (Figure 14). The synthetic strategy depends on the use of optically pure starting materials and follow previously published procedures for synthesis of racemic and/or (*R*)-crizotinib (see de Koning (2011) Organic Process Research & Development, 15: 1018-1026³⁸, which is herein incorporated by reference in its entirety). Indeed, when evaluated in the MTH1 catalytic assay, the IC₅₀ value determined for the (*S*)-enantiomer of crizotinib indicated more than 100-fold higher potency for the (*S*)- versus (*R*)-enantiomer (Figure 11).

The inventors prepared and tested both the pure, clinically used (*R*)- as well as the so far unexplored (*S*)-enantiomer of crizotinib. Analysis of both enantiomers in the MTH1 catalytic assay suggested that the screening hit batch contained a racemic mixture as the inventors found that the pure (*S*)-enantiomer of crizotinib is a low nanomolar MTH1 inhibitor whereas the (*R*)-enantiomer gave IC₅₀ values in the micromolar range (Figure 11). Results were confirmed by direct binding assays (ITC) indicating a 16-fold higher affinity of the (*S*)-enantiomer for MTH1 (Figure 17).

To assess the antiproliferative activity of the (*S*)-enantiomer against human cancer cells, we conducted colony formation assays using the two breast cancer cell lines MCF-7 and MDA-MB-231 (Figure 12), the latter bearing a KRAS mutation (G13D). At 10 μM concentration both the (*R*)- and (*S*)-enantiomer significantly decreased the growth of both cell lines in comparison to DMSO-treated cells.

Consistent with these data, the (*S*)-enantiomer of crizotinib efficiently inhibited colony formation of SW480 colon carcinoma and K-Ras mutated PANC1 pancreatic cancer cells, similar to SCH51344 (Figure 17A and 17C). Importantly, when the inventors treated normal human BJ skin fibroblasts with increasing concentrations of either (*R*)-crizotinib or the (*S*)-enantiomer of crizotinib, the inventors found that the clinically used (*R*)-enantiomer exhibited higher toxicity than the (*S*)-enantiomer of crizotinib on non-transformed cells (Figure 19). Thus, the antiproliferative effects observed for the (*S*)-enantiomer of crizotinib were not due to any increased non-specific cytotoxicity. The inventors also tested another two ALK and three commercially available Met inhibitors for their ability to inhibit MTH1, but no other compound displayed comparable potency (data not shown). Considering the published c-Met K_i value for the (*S*)-enantiomer of crizotinib, the data of the present invention suggest that the (*S*)-enantiomer of crizotinib is at least 4-fold more selective for MTH1 (Cui (2011) Journal of Medicinal Chemistry 54, 6342-6363). To exclude that the antiproliferative effects of the (*S*)-enantiomer of crizotinib are mediated by inhibition of the potential off-target c-Met the inventors treated SW480 cells with a specific, low nanomolar c-Met inhibitor, but did not detect significant effects on proliferation (Figure 20). Thus, the (*S*)-enantiomer of crizotinib is a novel and potent MTH1 inhibitor with antiproliferative activity against Ras transformed cancer cells.

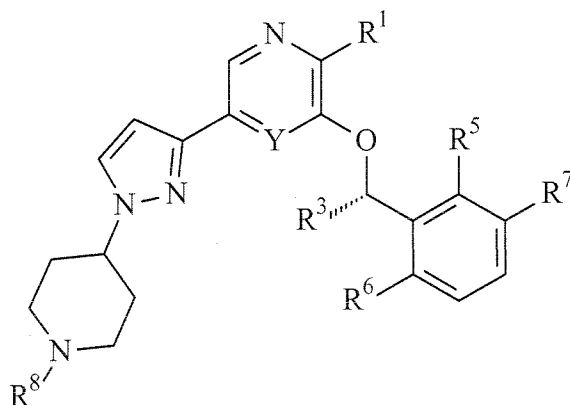
Since MTH1 is thought to sanitise the nucleotide pool of oxidised nucleotides including 8-oxo-dGTP, thereby preventing its incorporation into DNA, the inventors reasoned that inhibition of MTH1 enzymatic function should increase the content of 8-oxo-guanine in DNA. Consequently, this should in turn activate DNA repair mechanisms and induce the formation of single strand breaks due to activated base excision repair (BER) (Rai (2009) Proceedings of the National Academy of Sciences 106, 169-174). To test this the inventors investigated the (*S*)-enantiomer of crizotinib and SCH51344 in an alkaline comet assay, which reveals DNA single strand breaks by single cell electrophoresis. The inventors found that both the (*S*)-enantiomer of crizotinib as well as SCH51344, but not (*R*)-crizotinib, yielded a significant tail moment in the comet assay (Figure 18A). At the same time, staining for 53BP1, a specific marker for DNA damage, was increased, which was in line with the effect observed for anti-MTH1 siRNA (Figures 18B and 21).

Example 4: Preferred structural features of aminoheteroaryl-based MTH1 inhibitors

To determine the molecular requirements for the inhibitors to bind MTH1 the inventors cocrystallised both (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib with MTH1. The structure revealed that an unfavourable eclipsed conformation of the methyl group at the chiral centre and the halogen substituents on the benzyl ring may reduce the energetic favourability of binding (*R*)-crizotinib in the active site (Figures 18C, D, 22, 23, and Tables 3, 4). This observation is also supported by the ITC data which suggest that the difference in binding between (*R*)-crizotinib and the (*S*)-enantiomer of crizotinib is entirely entropic and therefore not due to different binding interactions with the protein.

As noted above, a cocrystal structure of the (*S*)-enantiomer of crizotinib bound to human MTH1 was generated (see Figure 15). On the basis of the cocrystal structure of the (*S*)-enantiomer of crizotinib bound to human MTH1, the MTH1 inhibitor pharmacophore model as shown in Figure 16 was developed. This pharmacophore model illustrates the preferred structural features of aminoheteroaryl compound-based MTH1 inhibitors. In particular, as shown in Figure 16, the MTH1 inhibitor pharmacophore comprises a 5- or 6-membered heteroaromatic ring with one hydrogen bond acceptor (e.g. -NH₂, -RNH, -RNR, -OH, -OR, -SH, -SR) and one hydrogen bond donor (e.g. -NH₂, -RNH, -OH, -SH) in ortho position to each other. The hydrogen bond acceptor bears preferably lipophilic benzyl substituents such as halogenated benzyis. The benzyl substituent preferably has an additional methyl group at the CH₂-carbon. With regard to the resulting chiral centre, (*S*)-configuration is preferred with regard to MTH1 inhibition. In para position to the main heteroaromatic ring additional aryl substituents may be introduced such as 5-membered heteroaromatic rings (e.g. pyrazol). The 5-membered ring preferably bears an aliphatic or cyclic substituent featuring a positively ionizable functional group which may also be capable of acting as a hydrogen bond donor (e.g. aminopropyl, piperidine, piperazine).

This pharmacophore model is in line with the following Formula (1) of an MTH1 inhibitor, which is an (*S*)-enantiomer of an aminoheteroaryl compound.



Formula (1)

wherein:

R^1 is $-NH_2$, $-NR^2H$, $-OH$ or $-SH$;

R^2 is C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl;

R^3 is C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl or cyclopropyl;

Y is N or CR^4 ;

R^4 is hydrogen, halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl or C_{3-6} cycloalkyl;

each R^5 , R^6 and R^7 is independently fluorine, chlorine, bromine or iodine;

R^8 is hydrogen or $-A-B_n-X$, wherein

A is a single bond, $-C(=O)-$ or $-C(=O)CH_2-$;

B is C_{1-4} alkylene, C_{2-4} alkenylene or $-(OCH_2CH_2)-$;

n is 0, 1, 2, 3, 4 or 5, and

X is $-NHR^2$; $-NH_2$; $-SH$; $-OH$ or O-alkyl;

Table 2. Isothermal titration calorimetry

	(<i>R</i>)-crizotinib	(<i>S</i>)- enantiomer of crizotinib	SCH51344
Molar ratio (n)	0.72	0.76	0.99
ΔH (cal/mol) [#]	-7306 ± 66	-7328 ± 38	-6479 ± 45
$T\Delta S$ (cal/mol)	+710	2330	3170
K_d (nM) [#]	781 ± 39	48 ± 3.9	49 ± 5.6

Data were measured at 15°C in 50 mM Tris-HCl pH 7.8, 150 mM NaCl.

[#]Error given in the table represent the error of the nonlinear least squares fit to the experimental data.

Table 3. Crystallization of MTH1 complexes

	MTH1 : (<i>R</i>)-crizotinib	MTH1 : (<i>S</i>)-enantiomer of crizotinib
Reservoir solution	30% PEG4000, 0.2 M (NH ₄) ₂ SO ₄	24% PEG4000, 0.2 M (NH ₄) ₂ SO ₄
Volume of protein : Volume of reservoir (nL)	50 : 100	50 : 100
Temperature (°C)	4	20

Table 4. Data Collection and Refinement Statistics

Dataset	MTH1 : (<i>R</i>)-crizotinib	MTH1 : (<i>S</i>)-enantiomer of crizotinib
Unit Cell (<i>a</i> , <i>b</i> , <i>c</i> (Å))	36.2, 60.0, 66.9	36.2, 60.0, 67.0
Spacegroup	<i>P</i> 22 ₁ 2 ₁	<i>P</i> 22 ₁ 2 ₁
Number of molecules / asymmetric unit	1	1
Data Collection		
Beamline	Diamond I04	Diamond I02
Resolution range ^a (Å)	44.64-1.65 (1.68-1.65)	36.20-1.20 (1.22-1.20)
Completeness ^a (%)	99.8 (97.4)	99.6 (99.8)
Multiplicity ^a	4.2 (3.2)	3.9 (4.0)
<i>R</i> _{merge} ^a (%)	0.077 (0.369)	0.054 (0.566)
<1/σ(<i>I</i>)> ^a	8.2 (2.6)	10.4 (2.2)
Refinement		
<i>R</i> factor (%)	16.7	15.6
<i>R</i> _{free} (%)	22.7	19.2
Rmsd bond length (Å) (angle (°))	0.009 (1.34)	0.008 (1.30)

^aValues in parentheses are for the highest resolution shell.

Example 5: Mouse Xenograft Study

To explore the *in vivo* potential of the (*S*)-enantiomer of crizotinib to abrogate tumour growth the inventors performed a mouse xenograft study using SW480 cells indicating that the (*S*)-enantiomer of crizotinib is able to impair tumour progression as shown by a reduction in tumour volume of more than 50% (Figure 18E). The (*S*)-enantiomer was well-tolerated as

animals behaved normally and no significant change in haematological parameters (Table 5) or body weight was observed ($18.8\text{mg} \pm 1.2$ (S)-crizotinib vs $19.1\text{mg} \pm 2.0$ vehicle group).

In summary the data suggest MTH1 is a critical component of Ras-transformed cells that can be readily targeted by drug-like small molecule inhibitors. It is well established that cancer cells are subject to high levels of oxidative stress due to increased proliferation leading to production of ROS as a result of mitochondrial respiration. The nucleotide pool represents a major target of ROS and oxidation of DNA bases contributes significantly to mutations and DNA damage. Consequently, tumour cells which bear a considerable amount of genetic aberrations and concomitant defects in DNA repair mechanisms are particularly sensitive to ROS-induced DNA damage. By removing oxidised nucleotides and thus maintaining nucleotide pool homeostasis, MTH1 relieves cancer cells of proliferative stress and is therefore a potential target for antitumoural compounds. Indeed, MTH1 levels are increased in Ras-expressing cancers (Figure 24) ranging from lung cancer (Speina (2005) Journal of the National Cancer Institute 97, 384-395; Kennedy (1998) FEBS Letters 429, 17-20) to renal carcinoma (Okamoto (1996) Int J Cancer 65, 437-441) indicating that there is a connection between oncogenic transformation and oxidative stress. This is also supported by the fact that SCH51344 was also shown to prevent growth of fibroblasts infected with a variety of different oncogenes such as v-abl prompting further exploration of a potential global role of MTH1. Remarkably, MTH1 deficiency in knockout mice confers a mild phenotype suggesting there is a potential therapeutic window for MTH1 inhibitors (Tsuzuki (2001) Proceedings of the National Academy of Sciences 98, 11456-11461). The identification of SCH51344 as a direct and functional inhibitor of MTH1 reveals genome integrity-related proteins as a new and druggable target class. Furthermore, our finding that the (S)-enantiomer of crizotinib, a clinically approved kinase inhibitor applied to patients with ALK aberrations, is a first-in-class low nanomolar MTH1 inhibitor that provides benefit in a K-Ras-positive colon carcinoma xenograft model may open a new therapeutic option in the treatment of cancer.

Table 5. SCID mouse hematology and liver/heart/kidney parameters

Test	(S)-enantiomer of crizotinib	(S)-enantiomer of crizotinib	p-value
Name (test unit)	(mean $\pm$ SD)	Fold of control	(t test)
WBC ($10^9/\text{l}$)	1.90 ± 0.64	1.08 ± 0.35	0.33

RBC (10 ¹² /l)	9.51 ± 0.37	1.00 ± 0.04	0.48
Neutrophils (10 ⁹ /l)	1.50 ± 0.51	1.24 ± 0.43	0.15
Lymphocytes (10 ⁹ /l)	0.23 ± 0.06	0.67 ± 0.19	0.11
Monocytes (10 ⁹ /l)	0.16 ± 0.11	0.95 ± 0.51	0.42
MCV (fl)	43.02 ± 1.07	1.01 ± 0.02	0.26
MCH (pg)	14.07 ± 0.30	1.0 ± 0.02	0.38
MCHC (g/l)	327 ± 4.90	0.98 ± 0.02	0.019
Thrombocytes (10 ⁹ /l)	828 ± 160	0.84 ± 0.16	0.21
HBG (g/l)	134 ± 6.25	0.99 ± 0.046	0.4
p-Creatinine (uM)	18.6 ± 4.24	0.93 ± 0.21	0.15
p-ASAT (mCat/l)	1.10 ± 0.32	0.72 ± 0.21	0.068
P-ALAT (mCat/l)	0.26 ± 0.07	0.81 ± 0.21	0.15

Mouse hematology and liver/heart/kidney parameters comparing treatment versus controls. SCID mice were subcutaneously administered vehicle or the (*S*)-enantiomer of crizotinib (25mg/kg) for 35 days. Blood samples were obtained by orbital bleeding (under anaesthesia), blood parameters were analysed using whole blood and ASAT, ALAT and creatinine were analysed in EDTA collected plasma by the Karolinska Universitetslaboratoriet, Clinical Chemistry. The mean values of white blood cells (WBC), red blood cells (RBC), neutrophils, lymphocytes, monocytes, mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) from the different groups are presented in the table. The results did not show any significant differences in the hematology parameters or the liver/heart/kidney parameters between control and treated groups apart from a minor change in MCHC.

Example 6: Stable knockdown of MTH1 impairs colony formation of SW480 cells

To corroborate our finding that MTH1 gene silencing by anti-MTH1 siRNA impairs viability of SW480 colon carcinoma cells expressing mutant KRAS, we also created stable cell lines expressing shRNA targeting either eGFP (control) or MTH1 (Rai (2009) Proceedings of the National Academy of Sciences 106, 169-174). Consistent with previous results stable knockdown of MTH1 significantly reduced colony formation of SW480 cells (Figure 26).

Example 7: Global cellular target profile of (*S*)-crizotinib

To determine the specificity of the (*S*)-enantiomer of crizotinib to target the MTH1 protein in cells, a chemical proteomics drug pull-down was performed using shotgun mass spectrometry as described above. For immobilization of (*S*)-crizotinib, derivatisation of (*S*)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)-5-(1-(piperidin-4-yl)-1H-pyrazol-3-yl)pyridin-2-amine was carried out as described for CeMM-146 to yield (*S*)-5-(1-(1-(3-aminopropyl)piperidin-4-yl)-1H-pyrazol-3-yl)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-2-amine (Figure 27).

Chemoproteomic analyses were performed as shown using SW480 colon carcinoma cells which express an oncogenic KRAS mutant. Results are presented in Table 6:

Table 6: Global cellular target profile of (S)-crizotinib

PreyGene	AvgP	SC.Ex p.1-1	SC.Ex p.1-2	SC.Ex p.2-1	SC.Ex p.2-2	Sum	SC.Ne gCtrl. 1-1	SC.Ne gCtrl. 1-2	SC.Ne gCtrl. 2-1	SC.Ne gCtrl. 2-2	Sum
NUDT1	0.9997	32	32	23	30	117	4	2	2	2	10
PLIN3	0.8429	4	3	3	3	13	0	0	1	0	1
DDX3X	0.7764	2	2	1	2	7	0	0	0	0	0
ANP32A	0.5951	1	1	1	3	6	0	0	0	0	0
KPNB1	0.5773	7	6	1	0	14	0	0	2	0	2
ANP32B	0.5116	0	1	4	4	9	0	0	0	0	0
UBA1	0.4997	15	15	0	0	30	0	0	2	2	4
PDIA3	0.4952	8	7	0	0	15	0	0	1	0	1
TLN1	0.4939	7	7	0	0	14	0	0	0	1	1
GARS	0.4818	6	7	0	0	13	0	0	2	0	2
PGAM1	0.4721	6	6	0	0	12	0	0	2	0	2
AHCY	0.4716	4	4	0	0	8	0	0	0	0	0
HSPH1	0.4697	7	5	0	0	12	0	0	0	0	0
SET	0.4696	3	3	4	3	13	0	0	2	3	5
SARS	0.4649	4	4	0	0	8	0	0	0	0	0
MDH1	0.4615	5	6	0	0	11	0	0	0	2	2
TARS	0.4614	5	4	0	0	9	0	0	0	0	0
ADK	0.4613	4	3	0	0	7	0	0	0	0	0
ANXA3	0.461	4	4	0	0	8	0	0	0	0	0
TXN	0.4587	4	5	2	1	12	0	0	1	3	4
IQGAP2	0.4584	9	9	0	0	18	0	0	0	0	0
PARK7	0.4547	6	7	0	0	13	0	0	0	0	0
PAICS	0.4488	7	5	0	0	12	0	0	2	1	3
NME1	0.4442	6	4	0	0	10	0	0	0	0	0
MDH2	0.4441	5	5	0	0	10	0	0	0	2	2
PDIA6	0.4427	3	4	0	0	7	0	0	0	0	0
COTL1	0.4372	5	4	0	0	9	0	0	0	0	0
ATIC	0.4369	3	4	0	0	7	0	0	0	0	0
PSMD2	0.4359	6	5	0	0	11	0	2	2	0	4
CDC37	0.4343	3	3	0	0	6	0	0	0	0	0
ANXA5	0.4332	9	6	0	0	15	0	0	2	2	4
PSMC3	0.4297	3	3	0	0	6	0	0	0	0	0
RUUBL2	0.4297	3	3	0	0	6	0	0	0	0	0
RBM39	0.4214	4	3	0	0	7	0	0	0	0	0
TPM3	0.4204	2	3	0	0	5	0	0	0	0	0
TPM4	0.4193	5	2	0	0	7	0	0	0	0	0
RCN1	0.4191	4	4	0	0	8	0	0	0	2	2
STMN1	0.4156	3	3	0	0	6	0	0	0	0	0
ARHGDIA	0.4128	2	3	0	0	5	0	0	0	0	0
ANXA2	0.41	13	13	0	0	26	0	0	5	6	11
EIF4H	0.4095	3	2	0	0	5	0	0	0	0	0
MAPRE1	0.4084	2	3	0	0	5	0	0	0	0	0
PHGDH	0.4081	5	7	0	0	12	0	0	2	2	4
FSCN1	0.4044	2	3	0	0	5	0	0	0	0	0
PPP2R1A	0.4042	3	3	0	0	6	0	0	0	0	0

PreyGene	AvgP	SC.Ex p.1-1	SC.Ex p.1-2	SC.Ex p.2-1	SC.Ex p.2-2	Sum	SC.Ne gCtrl. 1-1	SC.Ne gCtrl. 1-2	SC.Ne gCtrl. 2-1	SC.Ne gCtrl. 2-2	Sum
KRT14	0.4038	4	2	0	0	6	0	0	0	0	0
GPI	0.3986	4	5	0	0	9	0	0	1	2	3
P4HB	0.3981	3	4	0	0	7	0	0	1	1	2
SND1	0.3957	3	5	0	0	8	0	0	0	0	0
EIF1AY	0.3949	2	3	0	0	5	0	0	0	0	0
FASN	0.394	8	8	0	0	16	0	0	0	0	0
CACYBP	0.392	2	2	0	0	4	0	0	0	0	0
PSAT1	0.3916	3	3	0	0	6	0	0	1	0	1
CYCS	0.391	2	3	0	0	5	0	0	0	0	0
S100A4	0.3894	3	2	0	0	5	0	0	0	0	0
SOD1	0.3882	3	3	0	0	6	0	0	0	1	1
CFL1	0.3871	12	11	1	0	24	0	0	7	5	12
RUVBL1	0.3864	2	3	0	0	5	0	0	0	0	0
PFDN2	0.3862	2	2	0	0	4	0	0	0	0	0
SERPINH1	0.3843	5	6	0	0	11	0	0	1	3	4
HMGB1P1	0.3829	2	2	0	0	4	0	0	0	0	0
NPC2	0.3813	2	2	0	0	4	0	0	0	0	0
PPIB	0.381	2	2	0	0	4	0	0	0	0	0
LUC7L2	0.3775	3	3	0	0	6	0	0	0	1	1
S100A11	0.3773	4	5	0	0	9	0	0	1	2	3
TALDO1	0.3739	2	2	0	0	4	0	0	0	0	0
PPP1CA	0.3721	2	2	0	0	4	0	0	0	0	0
PTMA	0.3707	2	2	0	0	4	0	0	0	0	0
DPYSL2	0.3678	6	4	0	0	10	0	0	2	2	4
HARS	0.3676	3	3	0	0	6	0	0	2	0	2
KRT1	0.3569	26	24	5	3	58	9	8	6	3	26
HSPA9	0.3567	2	3	0	0	5	0	0	0	0	0
ARF4	0.352	3	3	0	0	6	0	0	2	0	2
TMSB10	0.3518	2	2	0	0	4	0	0	0	0	0
PSMD12	0.35	2	2	0	0	4	0	0	0	0	0
CAD	0.3483	2	3	0	0	5	0	0	0	1	1
FARSB	0.3413	3	2	0	0	5	0	0	0	0	0
MIF	0.3362	0	1	2	2	5	0	1	1	1	3
C1QBP	0.33	3	3	0	0	6	0	0	1	1	2
PEBP1	0.3266	3	3	0	0	6	0	0	1	1	2
PGD	0.3262	4	2	0	0	6	0	0	1	1	2
PRDX2	0.3255	4	4	0	0	8	0	0	2	1	3
09-Sep	0.323	1	2	0	2	5	1	0	1	0	2
MANF	0.3221	2	1	0	0	3	0	0	0	0	0
CTSZ	0.3217	2	2	0	0	4	0	0	0	1	1
HSD17B4	0.3179	2	3	0	0	5	0	0	0	0	0
PCNA	0.3137	2	1	0	0	3	0	0	0	0	0
NQO1	0.3104	2	1	0	0	3	0	0	0	0	0
CAP1	0.3081	3	2	0	0	5	0	0	0	2	2
RAN	0.3036	5	6	0	0	11	0	0	3	2	5
LIN28A	0.3021	2	2	2	0	6	2	0	1	0	3
DCTN2	0.2964	2	3	0	0	5	0	1	0	1	2
07-Sep	0.2962	3	3	0	0	6	0	0	1	2	3
HNRNPA2 B1	0.2944	2	2	0	2	6	1	1	0	1	3
ACTN4	0.2923	4	4	0	0	8	0	0	3	2	5
ACOT7	0.2785	1	2	0	0	3	0	0	0	0	0
MTHFD1	0.2666	2	2	0	0	4	0	0	0	0	0
SRP9	0.2561	2	1	0	2	5	2	0	1	0	3
CRABP2	0.2533	4	1	0	0	5	0	0	0	2	2

YARS	0.2511	2	1	0	0	3	0	0	0	0	0
PGK1	0.2466	14	12	0	0	26	0	0	9	6	15
DYNC1H1	0.2458	13	13	2	2	30	1	2	3	2	8
TGM2	0.2455	6	5	0	0	11	0	0	3	3	6
CAPRN1	0.2355	2	2	0	0	4	0	0	0	1	1
HIST1H1E	0.2298	0	2	0	2	4	0	3	0	0	3
MSN	0.2291	11	11	0	0	22	0	0	7	6	13
ALDOA	0.2283	12	12	2	0	26	0	0	8	6	14
HSP90B1	0.228	13	8	0	0	21	0	0	8	5	13
SLC1A5	0.227	1	0	3	2	6	2	2	0	1	5
NAP1L1	0.2244	1	2	0	0	3	0	0	1	0	1
ENO1	0.2209	29	28	7	7	71	3	3	17	18	41
PDXK	0.2207	3	0	0	0	3	0	0	0	0	0
ACTN1	0.2142	3	1	0	0	4	0	0	0	0	0
FAU	0.2109	2	4	0	2	8	2	1	1	1	5
COPB1	0.2093	1	3	0	0	4	0	0	0	0	0
SUB1	0.206	5	5	2	4	16	0	2	3	3	8
EIF3H	0.2043	3	0	0	0	3	0	0	0	0	0
DBF4B	0.2	0	0	2	0	2	0	0	0	0	0
PRDX6	0.1992	8	8	0	0	16	0	0	5	5	10
IDH1	0.1991	3	0	0	0	3	0	0	0	0	0
KRT9	0.1979	18	15	4	6	43	8	10	2	0	20
BLVRB	0.1915	0	2	0	0	2	0	0	0	0	0
TPD52L2	0.191	0	2	0	0	2	0	0	0	0	0
HN1L	0.1898	0	2	0	0	2	0	0	0	0	0
HPRT1	0.1891	2	0	0	0	2	0	0	0	0	0
ARF3	0.1881	2	0	0	0	2	0	0	0	0	0
PSMB4	0.1875	2	0	0	0	2	0	0	0	0	0
TXNDC17	0.1862	2	3	0	0	5	0	0	2	1	3
GDI2	0.1854	4	0	0	0	4	0	0	2	1	3
CTSB	0.1845	0	2	0	0	2	0	0	0	0	0
ERP29	0.1841	2	0	0	0	2	0	0	0	0	0
SLC2A3	0.1834	3	0	0	0	3	0	0	0	0	0
TAGLN2	0.1795	6	5	0	0	11	0	0	4	3	7
APEX1	0.1792	2	0	0	0	2	0	0	0	0	0
TOMM34	0.1791	0	2	0	0	2	0	0	0	0	0
LDHA	0.176	14	11	0	2	27	0	0	7	9	16
COPB2	0.1753	2	4	0	0	6	2	2	0	0	4
PRMT1	0.1736	2	0	0	0	2	0	0	0	0	0
GLOD4	0.1735	2	0	0	0	2	0	0	0	0	0
RPN1	0.1727	0	3	0	0	3	0	2	0	0	2
UBB	0.1726	3	4	1	2	10	1	2	1	1	5
GANAB	0.1726	3	0	0	0	3	0	0	0	0	0
TPI1	0.1723	14	18	6	5	43	0	0	12	11	23
ST13	0.169	2	2	0	0	4	0	0	2	1	3
ALDOC	0.1677	2	2	0	0	4	0	0	2	1	3
STIP1	0.1665	1	2	0	0	3	0	0	2	0	2
PSMC2	0.1654	0	2	0	0	2	0	0	0	0	0
PSMD11	0.1638	2	0	0	0	2	0	0	0	0	0
TXNDC5	0.1605	0	2	0	0	2	0	0	0	0	0
GSTP1	0.156	6	3	0	0	9	0	0	3	3	6
GEMIN4	0.1509	0	1	2	0	3	2	1	0	0	3
PSMA4	0.1477	1	1	2	1	5	2	0	1	1	4
WDR1	0.1469	0	2	0	0	2	0	0	0	0	0
PFN1	0.1469	9	9	1	3	22	0	2	6	6	14
ABCE1	0.1467	2	0	0	0	2	0	0	0	0	0
PDIA4	0.1432	2	0	0	0	2	0	0	0	0	0
EIF5A	0.1427	6	7	0	0	13	0	0	6	3	9

XRCC5	0.1426	2	0	0	0	2	0	0	0	0	0
DYNLRB1	0.1409	2	2	2	2	8	2	1	1	1	5
CARS	0.1394	0	2	0	0	2	0	0	0	0	0
FKBP4	0.1375	3	2	0	0	5	0	0	2	2	4
FABP3	0.1374	4	3	0	0	7	0	0	2	3	5
PKM	0.1356	23	22	6	6	57	0	2	13	14	29
RARS	0.1343	5	6	3	0	14	2	3	1	2	8
PSMB6	0.1336	0	2	0	0	2	0	0	0	2	2
CAST	0.1312	2	0	0	0	2	0	0	0	0	0
PRKCSH	0.1291	2	0	0	0	2	0	0	1	0	1
GSN	0.1286	0	2	0	0	2	0	0	0	0	0
DSTN	0.1269	0	2	0	0	2	0	0	2	0	2
YWHAH	0.1244	1	2	0	0	3	0	0	3	0	3
CNN2	0.1239	2	0	0	0	2	0	0	1	1	2
TKT	0.1231	5	7	0	0	12	0	0	4	5	9
YWHAE	0.1176	10	10	0	0	20	0	2	6	6	14
EZR	0.1136	4	6	0	0	10	0	0	5	3	8
PSMD1	0.1117	0	2	0	0	2	0	0	0	0	0
PDCD6IP	0.1109	2	0	0	0	2	0	0	0	0	0
AIMP1	0.1089	0	0	2	0	2	0	1	1	2	4
ACLY	0.1061	2	0	0	0	2	0	0	0	0	0
EIF4A1	0.1044	4	5	0	0	9	0	1	4	2	7
ATP2A2	0.1039	2	0	0	0	2	0	0	0	0	0
COPA	0.1038	0	2	0	0	2	0	0	0	0	0
HNRNPAB	0.1024	1	2	0	0	3	2	1	0	0	3
CLTA	0.1023	2	0	4	2	8	2	3	2	1	8
PLOD3	0.1017	2	0	0	0	2	0	0	1	0	1
IQGAP1	0.1001	6	4	0	0	10	0	2	2	5	9
GART	0.0981	0	2	0	0	2	0	0	0	0	0
HINT1	0.0956	0	2	0	3	5	1	0	2	3	6
HSPD1	0.0865	8	8	0	0	16	0	0	7	5	12
PFAS	0.0861	0	2	0	0	2	0	0	0	0	0
PSMA6	0.0839	3	3	0	1	7	2	1	1	1	5
NACA	0.0792	2	2	1	0	5	0	0	2	2	4
PSMA1	0.0751	0	2	0	0	2	0	1	1	1	3
EEF2	0.0725	15	15	0	0	30	0	0	11	11	22
RPS28	0.0702	2	3	1	3	9	3	2	1	1	7
PRDX1	0.0697	6	6	0	2	14	0	0	4	6	10
PDCD5	0.0686	2	2	0	0	4	0	0	2	2	4
CALM2,C ALM1,CA LM3	0.063	2	2	0	0	4	0	2	0	2	4
RPS20	0.0628	0	2	2	3	7	2	3	2	0	7
RANBP1	0.0579	2	2	0	0	4	0	0	2	2	4
CCT6A	0.053	9	8	0	2	19	3	3	4	3	13
ARFGEF2	0.051	0	2	2	3	7	4	3	0	0	7
STRAP	0.0503	4	4	0	3	11	3	3	2	0	8
XPO7	0.0419	5	4	6	4	19	3	6	2	2	13
GAPDH	0.0389	17	14	4	4	39	2	4	10	8	24
IPO5	0.0381	4	4	4	6	18	4	4	2	3	13
PSMA7	0.0354	2	2	1	2	7	2	2	1	1	6
CALR	0.0304	0	2	0	0	2	0	0	2	2	4
RPL21	0.0299	2	2	2	1	7	1	2	2	1	6

HIST1H4J, HIST1H4D ,HIST1H4 K,HIST1H 4C,HIST2H 4A,HIST4 H4,HIST1 H4E,HIST1 H4A,HIST 1H4B,HIST 1H4I,HIST 2H4B,HIST 1H4H,HIS T1H4L,HIS T1H4F	0.0286	2	3	0	0	5	2	2	2	0	6
ACTB	0.0283	19	18	6	3	46	3	3	12	12	30
EIF5B	0.0275	3	2	0	2	7	2	0	2	2	6
RPL38	0.0262	4	2	2	3	11	2	4	1	2	9
RBP1	0.0261	3	2	0	0	5	0	0	3	3	6
STAU1	0.0255	2	2	0	3	7	3	3	0	2	8
EIF3G	0.0243	2	2	0	2	6	2	2	0	2	6
SLC7A5	0.0239	3	2	2	2	9	3	2	0	2	7
PSMB3	0.0232	4	4	0	0	8	2	2	2	2	8
YWHAZ	0.0192	4	6	1	1	12	0	0	6	5	11
EIF3J	0.0189	3	2	0	2	7	2	3	2	0	7
LDHB	0.0184	9	8	2	3	22	0	0	7	9	16
YWHAB	0.0173	4	4	0	0	8	0	0	5	4	9
RPL36AL	0.0168	3	0	2	0	5	2	3	0	2	7
HNRNPK	0.0165	6	5	3	0	14	2	1	4	4	11
VCP	0.0163	15	14	3	4	36	4	5	6	9	24
CLIC1	0.0151	8	8	0	1	17	0	0	7	8	15
HDAC6	0.0145	2	2	0	1	5	2	1	1	1	5
RPL35A	0.0138	1	2	0	0	3	1	2	1	1	5
YWHAG	0.0136	3	3	1	2	9	0	0	4	4	8
SYNE1	0.0134	3	4	0	0	7	0	0	0	0	0
XPO1	0.0132	4	2	2	0	8	5	3	1	0	9
RPL27A	0.0111	7	2	3	2	14	3	3	4	3	13
PRKDC	0.0073	10	8	4	9	31	15	7	0	0	22
MAP4	0.0072	6	5	4	6	21	4	4	3	4	15
HNRNPU	0.0071	2	3	2	3	10	2	2	2	3	9
SSB	0.0068	2	2	0	0	4	2	1	2	1	6
KARS	0.0068	1	1	0	2	4	3	2	2	0	7
CCT3	0.0066	7	7	4	5	23	3	4	4	3	14
RPL35	0.0065	2	0	2	2	6	2	2	2	2	8
EIF3F	0.0064	3	2	0	0	5	3	2	2	0	7
RPL34	0.0057	0	2	0	0	2	2	0	2	2	6
RPS26	0.0053	3	3	3	4	13	3	3	3	3	12
02-Sep	0.0051	6	2	1	2	11	2	4	3	3	12
RPL29	0.005	0	0	2	2	4	2	2	2	2	8
YWHAQ	0.0042	6	6	0	0	12	0	0	7	7	14
MARS	0.0042	3	2	3	1	9	3	3	1	3	10
CCT4	0.0041	10	10	2	2	24	4	2	7	6	19
SYNE2	0.0039	2	0	0	0	2	0	0	0	0	0
HSPA5	0.0033	18	14	4	4	40	4	5	11	10	30
VDAC1	0.0033	5	3	7	5	20	6	7	3	2	18
THADA	0.0025	2	0	3	2	7	4	7	0	0	11
EIF2S1	0.0022	2	2	1	4	9	3	3	3	3	12
RPL27	0.002	4	5	0	3	12	3	3	4	3	13

RPLP1	0.0019	3	2	0	0	5	2	2	2	3	9
EIF3M	0.0019	2	2	0	0	4	2	1	2	2	7
EPDR1	0.0018	4	3	5	4	16	5	4	3	3	15
RPS23	0.0016	1	2	2	1	6	2	3	2	2	9
RPS13	0.0016	4	4	3	3	14	2	4	2	4	12
HIST1H2A B,HIST1H2 AE	0.0014	2	3	2	2	9	3	3	2	2	10
SLC2A1	0.0014	2	2	2	2	8	3	3	3	1	10
RPL14	0.0013	3	2	3	2	10	3	4	2	2	11
MYL6	0.0012	5	4	2	4	15	4	4	3	3	14
RPL19	0.0012	4	2	2	2	10	2	5	0	4	11
RPL9,RPL 9P8,RPL9P 7,RPL9P9	0.0011	2	2	1	2	7	3	4	2	1	10
RPL22	0.0011	3	3	1	1	8	4	2	2	2	10
EIF3D	0.0009	5	4	3	3	15	5	2	2	4	13
RPL31	0.0008	3	3	4	3	13	3	3	4	4	14
EIF3I	0.0008	3	2	0	0	5	3	3	2	1	9
MLST8	0.0008	7	7	6	8	28	8	7	3	3	21
EEF1D	0.0007	3	4	3	2	12	4	3	3	2	12
RPS9	0.0007	5	4	2	2	13	2	4	3	4	13
PA2G4	0.0005	10	11	4	4	29	5	5	6	7	23
KRT5	0.0004	5	5	1	0	11	6	3	3	2	14
RPL5	0.0004	3	3	4	3	13	5	5	2	2	14
RPL18	0.0004	4	4	5	6	19	6	4	3	6	19
PSMB2	0.0003	0	3	0	0	3	2	3	2	3	10
LARS	0.0003	5	6	3	3	17	7	3	2	3	15
HSPB1	0.0002	6	7	2	4	19	4	4	5	5	18
PSMB1	0.0002	2	2	0	0	4	3	3	1	2	9
RPL13A	0.0002	4	4	2	3	13	4	2	3	4	13
RPS11	0.0002	4	4	5	4	17	6	4	3	4	17
RPL30	0.0002	3	2	2	3	10	2	4	3	4	13
RPL18A	0.0002	3	3	4	2	12	4	3	4	4	15
KRT8	0.0001	12	8	3	4	27	6	7	5	6	24
XRCC6	0.0001	5	4	0	3	12	5	4	2	4	15
PSMB5	0.0001	4	4	3	4	15	5	4	4	3	16
RPS19	0.0001	8	7	3	6	24	5	6	5	4	20
CCT5	0.0001	6	4	0	0	10	3	3	5	5	16
RPS25	0.0001	6	6	5	5	22	4	5	4	6	19
PCBP2	0.0001	3	3	0	0	6	4	3	2	3	12
CCT7	0.0001	10	10	2	3	25	4	6	6	6	22
NA	0	69	67	53	61	250	75	69	61	63	268
NA	0	7	5	1	0	13	38	12	3	1	54
PSMD14	0	0	1	0	0	1	0	0	0	0	0
SLC9A3R1	0	1	0	0	0	1	0	0	0	0	0
TAX1BP3	0	1	0	0	0	1	0	0	0	0	0
MYL12B	0	4	3	3	2	12	3	3	4	4	14
SURF4	0	1	1	1	1	4	6	3	1	1	11
PSMD3	0	0	1	0	0	1	0	0	0	0	0
SYNCRIP	0	7	8	3	5	23	10	8	7	7	32
HNRNPCL 1	0	1	0	0	0	1	0	0	0	0	0
PFDN1	0	0	1	0	0	1	0	0	0	0	0
GGCT	0	0	1	0	0	1	0	0	0	0	0
FLNB	0	28	29	9	11	77	21	19	14	16	70
BANF1	0	1	1	0	0	2	0	0	0	0	0
IPO7	0	12	9	6	5	32	8	9	5	6	28

MT2A	0	1	1	0	0	2	0	0	0	0	0
CSTB	0	1	0	0	0	1	0	0	0	0	0
CAPNS1	0	0	1	0	0	1	0	0	0	0	0
RPLP2	0	4	4	6	7	21	8	8	5	6	27
RPLP0	0	12	10	6	10	38	13	12	10	12	47
KRT18	0	7	3	5	3	18	4	6	5	5	20
NPM1	0	2	2	1	1	6	3	4	4	3	14
CTSD	0	1	1	0	0	2	0	0	0	0	0
TUBB	0	18	20	10	9	57	19	22	18	22	81
EPRS	0	3	4	0	2	9	5	6	1	2	14
HSP90AA1	0	19	21	6	7	53	3	5	17	17	42
HSPA1A,H SPA1B	0	11	10	5	5	31	7	8	9	9	33
HSP90AB1	0	19	21	9	8	57	7	6	21	19	53
RPS17	0	2	3	0	0	5	3	3	5	3	14
RPSA	0	5	7	4	5	21	6	5	5	6	22
ENO2	0	1	0	0	0	1	1	0	5	5	11
SNRPA1	0	0	0	0	1	1	2	0	0	0	2
PARP1	0	0	1	0	0	1	0	0	0	0	0
HSPA8	0	16	18	8	9	51	11	14	11	16	52
PABPC1	0	7	10	7	5	29	10	11	6	8	35
IMPDH2	0	1	0	0	0	1	0	0	0	0	0
CKB	0	1	1	0	0	2	0	0	0	0	0
RNH1	0	0	1	0	0	1	0	0	0	0	0
KRT10	0	16	14	0	0	30	12	12	7	7	38
DARS	0	6	6	0	1	13	2	5	5	6	18
RPS2	0	6	6	5	5	22	9	8	6	5	28
HIST1H1B	0	1	0	0	0	1	1	1	1	1	4
CSDA	0	2	2	2	2	8	5	5	3	3	16
HSPA6	0	1	1	1	1	4	5	6	6	7	24
TCP1	0	11	8	4	4	27	8	4	7	6	25
RPL7	0	4	4	2	2	12	7	5	4	5	21
VCL	0	1	0	0	0	1	0	0	0	0	0
RPL17	0	3	4	3	3	13	4	4	3	5	16
NCL	0	13	14	8	9	44	12	14	15	14	55
HK1	0	1	0	0	0	1	0	0	0	0	0
EIF2S2	0	4	6	2	4	16	4	5	6	5	20
PTMS	0	1	1	0	0	2	0	0	0	0	0
FLNA	0	43	43	32	30	148	47	51	34	36	168
RPS3	0	9	9	7	8	33	8	8	5	7	28
EEF1B2	0	2	3	2	3	10	4	4	4	3	15
ACP1	0	5	5	4	3	17	5	6	6	5	22
RPS12	0	6	5	4	6	21	5	6	5	5	21
RPL13	0	6	6	4	6	22	7	7	6	7	27
PTBP1	0	1	0	0	0	1	0	0	0	0	0
EEF1G	0	7	9	9	7	32	9	10	10	10	39
RPL12	0	7	12	8	7	34	9	11	9	8	37
HSPA4	0	13	16	2	4	35	7	8	9	11	35
PHB	0	8	9	5	4	26	12	10	8	5	35
MYH9	0	32	33	15	18	98	18	19	22	24	83
FUS	0	1	0	0	0	1	1	0	0	0	1
KRT2	0	8	8	0	0	16	9	8	6	4	27
RPL4	0	8	4	5	5	22	5	6	8	8	27
SRP14	0	1	1	1	1	4	1	1	1	1	4
RPL3	0	5	5	4	4	18	5	7	6	6	24
EIF2S3	0	5	4	3	4	16	3	3	7	4	17
IARS	0	4	4	2	3	13	4	4	3	4	15
MTOR	0	9	10	9	11	39	24	19	3	4	50

RPS27	0	0	0	0	1	1	2	2	0	0	4
VDAC2	0	1	0	0	0	1	1	1	0	0	2
RPL28	0	4	2	0	0	6	6	4	3	4	17
RPS5	0	5	4	3	2	14	5	6	5	4	20
RPS10	0	7	5	5	3	20	7	7	7	8	29
MAP1B	0	21	20	14	12	67	26	25	23	22	96
PPT1	0	1	0	0	0	1	0	0	0	0	0
CCT8	0	8	8	4	4	24	5	4	6	6	21
CSEIL	0	11	12	6	7	36	14	14	5	4	37
EIF3B	0	3	3	2	3	11	5	4	5	4	18
EIF6	0	2	2	1	0	5	3	4	3	3	13
MTPN	0	0	1	0	0	1	0	0	0	1	1
EIF3E	0	2	2	2	2	8	3	4	3	2	12
RAB10	0	1	0	0	0	1	0	0	0	0	0
UBE2N	0	1	1	0	0	2	0	0	0	0	0
RPS3A	0	9	6	6	6	27	5	7	5	8	25
RPL26	0	3	3	2	2	10	3	5	6	4	18
RPL15	0	4	4	4	3	15	5	6	4	4	19
RPL37A	0	3	4	3	4	14	4	5	4	4	17
HSPE1	0	1	1	0	0	2	0	0	0	0	0
RPS7	0	5	7	2	2	16	7	5	7	5	24
PSMC5	0	1	1	0	1	3	1	1	0	0	2
RPS8	0	7	6	5	6	24	8	8	6	5	27
RPS16	0	6	4	6	5	21	6	7	5	4	22
RPS14	0	6	6	5	5	22	6	6	5	5	22
RPS18	0	8	7	4	8	27	6	6	7	9	28
TMSB4X	0	1	1	0	0	2	0	0	0	0	0
RPL7A	0	3	3	5	3	14	6	7	6	4	23
RPS4X	0	8	7	3	6	24	10	10	11	7	38
PPP2CB	0	1	1	0	0	2	0	0	0	0	0
ACTA2	0	2	1	0	0	3	3	2	7	10	22
RPL23A	0	3	3	2	3	11	5	3	5	4	17
RPS6	0	5	4	3	3	15	3	4	4	5	16
RAB1A	0	0	1	0	0	1	0	0	0	1	1
RPL23	0	5	5	4	4	18	5	5	5	6	21
RPL10A	0	5	4	3	4	16	4	4	3	5	16
RPL32	0	1	1	1	1	4	1	2	1	1	5
RPL11	0	3	4	3	4	14	5	5	5	4	19
RPL8	0	6	5	5	6	22	6	5	7	7	25
PPIA	0	13	13	4	3	33	8	6	10	10	34
FKBP1A	0	1	1	1	0	3	0	0	0	1	1
GNB2L1	0	12	13	9	11	45	13	14	12	14	53
YBX1	0	2	2	2	2	8	6	5	4	3	18
EEF1A1	0	23	21	8	8	60	12	11	16	16	55
TUBA1B	0	1	1	0	0	2	22	20	19	21	82
TUBA4A	0	22	20	14	12	68	18	16	16	17	67
TUBB4B	0	8	8	2	4	22	16	19	16	18	69
PAFAH1B 2	0	1	1	0	0	2	0	0	0	1	1
CCT2	0	9	11	3	3	26	6	5	9	6	26
RPL24	0	4	4	3	4	15	4	4	4	4	16
CLTC	0	16	14	5	7	42	16	17	12	10	55
EWSR1	0	0	1	0	0	1	1	0	0	0	1
RPL6	0	7	11	4	6	28	9	8	10	6	33
SSBP1	0	1	1	0	0	2	1	0	0	0	1
GFPT1	0	1	1	0	0	2	0	0	0	0	0
BAX	0	1	1	1	1	4	1	1	1	1	4
AHNAK	0	5	6	6	6	23	4	7	6	5	22

MTAP	0	1	1	0	0	2	0	0	0	0	0
G3BP1	0	2	2	3	2	9	4	4	4	2	14
PDAP1	0	1	1	0	0	2	0	0	0	0	0
TUBB2A	0	5	4	2	2	13	15	17	14	16	62
EIF3A	0	9	7	7	6	29	9	7	7	8	31
EIF4A2	0	1	1	0	1	3	2	2	4	3	11
FLNC	0	0	0	0	1	1	2	4	3	3	12
LASP1	0	0	1	0	0	1	0	0	0	2	2
PAFAH1B3	0	1	1	0	0	2	0	0	0	0	0
PCBP1	0	0	1	0	0	1	1	1	3	3	8
ACTBL2	0	1	1	0	0	2	0	0	4	4	8
KRT79	0	0	1	0	0	1	2	1	1	3	7
TUBA1A	0	12	11	9	10	42	21	20	18	20	79
RPS27L	0	0	1	0	0	1	0	2	2	2	6
KRT74	0	1	1	0	0	2	0	0	0	0	0
MYH14	0	1	1	0	0	2	0	0	0	0	0
SERBP1	0	8	10	5	6	29	8	7	10	8	33
GCN1L1	0	37	34	37	37	145	60	61	17	12	150
TNPO1	0	9	7	6	6	28	14	8	5	6	33
S100A16	0	1	0	0	0	1	1	0	0	0	1
EIF3C,EIF3CL	0	4	6	7	7	24	10	7	3	3	23
PHB2	0	9	8	3	5	25	14	11	8	7	40
TUBA1C	0	1	1	0	1	3	20	19	18	19	76
TUBB2B	0	4	3	1	2	10	16	18	15	17	66
TMEM109	0	1	1	0	1	3	2	1	0	1	4
NACAP1	0	0	0	1	0	1	0	0	0	0	0
OLA1	0	1	1	0	0	2	0	0	0	1	1
IGF2BP1	0	4	8	4	6	22	8	7	4	6	25
STOML2	0	5	4	2	2	13	8	8	2	3	21
EIF3L	0	9	7	6	4	26	9	11	7	8	35
NUDC	0	0	1	0	0	1	0	0	0	0	0
CNPY2	0	1	0	0	0	1	0	0	0	0	0

The results clearly indicate that MTH1 (gene name NUDT1) is the main target of (*S*)-crizotinib in SW480 cells, a colon carcinoma cell line expressing mutant KRAS highlighting the specificity of the compound.

Example 8: Anti-cancer specificity

To further evaluate the anti-cancer specificity of the (*S*)-enantiomer of crizotinib *in vitro*, various isogenic BJ fibroblast cell lines were treated with several concentrations of the (*S*)-enantiomer of crizotinib in colony formation assays. The investigated cell line panel comprised non-transformed (“wildtype”) cells, cells immortalized with telomerase (hTERT), cells transformed with hTERT and SV40 Large T antigen (SV40T), and cells transformed with hTERT, SV40T, and the KRAS mutant V12 (Figure 28).

The data suggest that (S)-crizotinib is more toxic to transformed cells than to wildtype cells or cells which are only immortalized by telomerase thus underscoring a cancer-specific effect.

Example 9: Inhibition of MTH1-catalytic activity by the (S)-enantiomer of crizotinib is not substrate-dependent

It is known that MTH1 does not only hydrolyse 8-oxo-dGTP but also is able to cleave another potentially mutagenic DNA precursor, 2-hydroxy-deoxyadenosine triphosphate (2-OH-dATP) (Fujikawa (1999) Journal of Biological Chemistry 274, 18201-18205). Therefore we investigated whether the (S)-enantiomer of crizotinib affects hydrolysis of 2-OH-dATP by MTH1 *in vitro* using the previously described luciferase-based PPiLight assay. As shown in Figure 29, the (S)-enantiomer of crizotinib also inhibits hydrolysis 2-OH-dATP by MTH1 in a dose-dependent manner.

Example 10: Effect of p53-/p21-status and mismatch repair pathways on the activity of the (S)-enantiomer of crizotinib

The tumor suppressor gene p53 is mutated or defective in a large number of various cancer types and can impact therapeutic outcome. Therefore we examined how p53 function and its downstream mediator p21 might affect the anticancer activity of the (S)-enantiomer of crizotinib in mutant KRAS HCT116 colon carcinoma cells. Furthermore, as inhibition of MTH1 is supposed to induce mispairing of base during DNA replication we sought to evaluate how deficiencies in mismatch repair genes such as MLH1 might alter sensitivity toward the (S)-enantiomer of crizotinib using the established HCT116 isogenic cell line system (Figure 30). HCT116 wildtype cells have functional p53 but are deficient in MLH1 whereas the derivative HCT116 +chr3 has a functional MLH1 gene due to chromosomal transfer.

The results indicate that p53 status and presence or absence of functional MLH1 do not affect the activity of the (S)-enantiomer of crizotinib. However, loss of p21 seems to increase the sensitivity of HCT116 cells toward treatment with the (S)-enantiomer of crizotinib.

Example 11: Induction of DNA damage as a result of MTH1 inhibition

Since MTH1 is thought to sanitise the nucleotide pool of oxidised nucleotides including 8-oxo-dGTP, thereby preventing its incorporation into DNA, we reasoned that inhibition of MTH1 enzymatic function should increase the content of 8-oxo-guanine in DNA. Indeed, treatment of BJ fibroblasts with (*S*)-, but not (*R*)-crizotinib, increased staining of an anti-8-oxo-guanine mouse monoclonal antibody. At the same time, staining for 53BP1, a specific marker for DNA damage, was increased, which was in line with the effect observed for anti-MTH1 siRNA (Figure 31).

Consequently, this should in turn activate DNA repair mechanisms and induce the formation of single strand breaks due to activated base excision repair (BER). To test this we investigated the (*S*)-enantiomer of crizotinib in an alkaline comet assay, which reveals DNA single strand breaks by single cell electrophoresis. Using SW480 cells we found that both (*S*)-crizotinib, but not (*R*)-crizotinib, yielded a significant tail moment in the comet assay. We also observed an increase of ATM phosphorylation indicating DNA damage pathway activation again demonstrating an (*S*)-crizotinib-specific effect (Figure 32).

Summary

Taken together, the data indicate that crizotinib, more preferably racemic crizotinib, and even more preferably the pure (*S*)-enantiomer of crizotinib are highly potent inhibitors of MTH1, an enzyme which has been linked to the development, progression and maintenance of RAS-driven cancer.¹³ This is of particular interest as a) small molecule inhibitors of MTH1 have not been reported so far, b) patients who are to be treated with crizotinib are selected by their c-Met-, or more preferably, ALK-expression status whilst not considering MTH1, and c) introduction of aminoalkyl substituents at the piperidine nitrogen of crizotinib is well tolerated with respect to both ALK and MTH1 inhibition. Thus, also compounds prepared by the procedures described in WO2008053157 are potent and bioavailable MTH1 inhibitors.

Based on the physiological effects of MTH1 suppression, MTH1 inhibitors are concluded to synergise with DNA damaging compounds, compounds which interfere with DNA repair mechanisms, or compounds which induce the production or inhibit the clearance of ROS. Furthermore, MTH1 inhibitors are concluded to exhibit synthetic lethality with tumours bearing lesions in DNA repair pathway genes or which produce considerable amounts of

ROS. For example, breast cancer cells deficient in the genes BRCA1 or BRCA2 which have been associated with repair of 8-oxo-G lesions¹⁷ are particularly sensitive towards oxidative DNA damage.¹⁸ Recently, it has been shown that MTH1 specifically assists RAS-induced tumours in preventing tumour-suppressive effects such as senescence whilst enabling maintenance and progression of the tumour. Therefore, MTH1 inhibition, may help in impairing tumour growth by abrogating mitogenic signalling, epithelial-mesenchymal transition (EMT), a hallmark of progressing and aggressive tumours, anoikis inhibition and PI3K/Akt-mediated pro-survival signalling.¹⁹ Recent data also suggests that MTH1 might be a promising target for adenocarcinomas expressing EGFR as the micro-RNA MiR-145 which suppresses both EGFR and MTH1 is downregulated in these tumours.³⁷ Reexpression of MiR-145 led to a downregulation of EGFR and MTH1 on both mRNA and protein level and impaired the growth of EGFR-positive cell lines. In summary, crizotinib and its enantiomers as well as structural derivatives thereof can be applied to the treatment of a variety of distinct tumour types apart from ALK/Met-driven lung cancer but also RAS-driven lung or breast cancer, colon cancer, pancreatic cancer, Ewing's sarcoma and many more.

The present invention refers to the following nucleotide and amino acid sequences:

1. Sequences relating to NUDT1/MTH1

1.1 NM_002452.3 → NP_002443.3 7,8-dihydro-8-oxoguanine triphosphatase isoform p18:

Description

Transcript Variant: This variant (1) encodes the predominant isoform (p18, also known as MTH1d). Variants 1, 2A, 3A, and 4A encode the same isoform.

SEQ ID No. 1: Nucleotide Sequence (471 nt) of NUDT1/MTH1, Isoform p18, Transcript Variant (1)

SEQ ID No. 2: Amino Acid Sequence (156 aa) of NUDT1/MTH1, Isoform p18, Transcript Variant (1)

1.2 NM_198948.1 → NP_945186.1 7,8-dihydro-8-oxoguanine triphosphatase isoform p18:

Description

Transcript Variant: This variant (2A) differs in the 5' UTR compared to variant 1. Variants 1, 2A, 3A, and 4A encode the same isoform (p18, also known as MTH1d).

SEQ ID No. 3: Nucleotide Sequence (471 nt) of NUDT1/MTH1, Isoform p18, Transcript Variant (2A)

SEQ ID No. 4: Amino Acid Sequence (156 aa) of NUDT1/MTH1, Isoform p18, Transcript Variant (2A)

- 1.3 NM_198949.1 → NP_945187.1 7,8-dihydro-8-oxoguanine triphosphatase isoform p22:

Description

Transcript Variant: This variant (2B) differs in the 5' UTR and 5' coding region compared to variant 1, resulting in translation initiation at an upstream ATG and an isoform (p22, also known as MTH1b) with a longer N-terminus compared to isoform p18. Variants 2B, 3B, and 4B encode the same isoform.

SEQ ID No. 5: Nucleotide Sequence (540 nt) of NUDT1/MTH1, Isoform p22, Transcript Variant (2B)

SEQ ID No. 6: Amino Acid Sequence (179 aa) of NUDT1/MTH1, Isoform p22, Transcript Variant (2B)

- 1.4 NM_198950.1 → NP_945188.1 7,8-dihydro-8-oxoguanine triphosphatase isoform p18:

Description

Transcript Variant: This variant (3A) differs in the 5' UTR compared to variant 1. Variants 1, 2A, 3A, and 4A encode the same isoform (p18, also known as MTH1d).

SEQ ID No. 7: Nucleotide Sequence (471 nt) of NUDT1/MTH1, Isoform p18, Transcript Variant (3A)

SEQ ID No. 8: Amino Acid Sequence (156 aa) of NUDT1/MTH1, Isoform p18, Transcript Variant (3A)

- 1.5 NM_198952.1 → NP_945190.1 7,8-dihydro-8-oxoguanine triphosphatase isoform p22:

Description

Transcript Variant: This variant (3B) differs in the 5' UTR and 5' coding region compared to variant 1, resulting in translation initiation at an upstream ATG and an isoform (p22, also known as MTH1b) with a longer N-terminus compared to isoform p18. Variants 2B, 3B, and 4B encode the same isoform.

SEQ ID No. 9: Nucleotide Sequence (540 nt) of NUDT1/MTH1, Isoform p22, Transcript Variant (3B)

SEQ ID No. 10: Amino Acid Sequence (179 aa) of NUDT1/MTH1, Isoform p22, Transcript Variant (3B)

- 1.6 NM_198953.1 → NP_945191.1 7,8-dihydro-8-oxoguanine triphosphatase isoform p18:

Description

Transcript Variant: This variant (4A) differs in the 5' UTR compared to variant 1. Variants 1, 2A, 3A, and 4A encode the same isoform (p18, also known as MTH1d).

SEQ ID No. 11: Nucleotide Sequence (471 nt) of NUDT1/MTH1, Isoform p18, Transcript Variant (4A)

SEQ ID No. 12: Amino Acid Sequence (156 aa) of NUDT1/MTH1, Isoform p18, Transcript Variant (4A)

- 1.7 NM_198954.1 → NP_945192.1 7,8-dihydro-8-oxoguanine triphosphatase isoform p22:

Description

Transcript Variant: This variant (4B) differs in the 5' UTR and 5' coding region compared to variant 1, resulting in translation initiation at an upstream ATG and an isoform (p22, also known as MTH1b) with a longer N-terminus compared to isoform p18. Variants 2B, 3B, and 4B encode the same isoform.

SEQ ID No. 13: Nucleotide Sequence (540 nt) of NUDT1/MTH1, Isoform p22, Transcript Variant (4B)

SEQ ID No. 14: Amino Acid Sequence (179 aa) of NUDT1/MTH1, Isoform p22, Transcript Variant (4B)

SEQ ID No. 15: Amino Acid Sequence of NUDT1/MTH1, p26 isoform

>sp|P36639|8ODP_HUMAN 7,8-dihydro-8-oxoguanine triphosphatase OS=Homo sapiens GN=NUDT1 PE=1 SV=3

SEQ ID No. 16: Amino Acid Sequence of NUDT1/MTH1, p21 isoform

>sp|P36639-3|8ODP_HUMAN Isoform p21 of 7,8-dihydro-8-oxoguanine triphosphatase OS=Homo sapiens GN=NUDT1

Primer Sequences:

SEQ ID No. 17: Primer Sequence for the Detection of NUDT1/MTH1

(Corresponding to bases 97-116 of the human MTH1 sequence published in Sakumi (1993) Journal of Biological Chemistry 268: 23524-30.))
5P-AGCCTCAGCGAGTTCTCCTG-3P

SEQ ID No. 18: Primer Sequence for the Detection of NUDT1/MTH1

(Corresponding to bases 248-266 of the human MTH1 sequence published in Sakumi (1993) Journal of Biological Chemistry 268: 23524-30.))
5P-GATCTGGCCACCTTGTGC-3P

2. Sequences relating to KRAS

2.1 NM_004985.3 → NP_004976.2 GTPase KRas isoform b precursor:

Description

Transcript Variant: This variant (b) is composed of five exons and lacks exon 4a which the longer transcript variant (a) includes. This predominant variant (b) has a cds that terminates in exon 4b and encodes isoform b.

SEQ ID No. 19: Nucleotide Sequence (567 nt) of KRAS, Isoform b precursor, Transcript Variant (b)

SEQ ID No. 20: Amino Acid Sequence (188 aa) of KRAS, Isoform b precursor, Transcript Variant (b)

2.2 NM_033360.2 → NP_203524.1 GTPase KRas isoform a precursor:

Description

Transcript Variant: This variant (a) is composed of six exons, including exon 4a, which the shorter transcript variant (b) lacks. This rare variant (a) has a cds that terminates in exon 4a and encodes a unique C-terminus, compared to isoform a.

SEQ ID No. 21: Nucleotide Sequence (570 nt) of KRAS, Isoform a precursor, Transcript Variant (a)

SEQ ID No. 22: Amino Acid Sequence (189 aa) of KRAS, Isoform a precursor, Transcript Variant (a)

KRAS Sequence Annotation

In this Sequence Annotation, common mutations of KRAS are indicated by the term "variant".

FT	CHAIN	1	186	GTPase KRas.
FT				/FTId=PRO_0000082641.
FT	INIT_MET	1	1	Removed; alternate.
FT	CHAIN	2	186	GTPase KRas, N-terminally processed.
FT				/FTId=PRO_0000326480.
FT	PROPEP	187	189	Removed in mature form.
FT				/FTId=PRO_0000281291.
FT	NP_BIND	10	17	GTP.
FT	NP_BIND	57	61	GTP.
FT	NP_BIND	116	119	GTP.
FT	REGION	166	185	Hypervariable region.
FT	MOTIF	32	40	Effector region.
FT	MOD_RES	1	1	N-acetylmethionine; in GTPase KRas;
FT				alternate.
FT	MOD_RES	2	2	N-acetylthreonine; in GTPase KRas, N-
FT				terminally processed.
FT	MOD_RES	186	186	Cysteine methyl ester.
FT	LIPID	180	180	S-palmitoyl cysteine.
FT	LIPID	186	186	S-farnesyl cysteine.
FT	VAR_SEQ	151	153	RVE -> GVD (in isoform 2B).

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FT				/FTId=VSP_011140.
FT	VAR_SEQ	165	189	QYRLKKISKEEKTPGCVKIKKCIIM -> KHKEKMSKDGGK
FT				KKKKSKTKCVIM (in isoform 2B).
FT				/FTId=VSP_011141.
FT	VARIANT	5	5	K -> E (in NS3).
FT				/FTId=VAR_065144.
FT	VARIANT	5	5	K -> N (in GASC; found also in a patient
FT				with Costello syndrome; exhibits only
FT				minor alterations in its in vitro
FT				biochemical behavior compared to wild-
FT				type protein).
FT				/FTId=VAR_064849.
FT	VARIANT	10	10	G -> GG (in one individual with AML;
FT				expression in 3T3 cell causes cellular
FT				transformation; expression in COS cells
FT				activates the Ras-MAPK signaling pathway;
FT				lower GTPase activity; faster GDP
FT				dissociation rate).
FT				/FTId=VAR_034601.
FT	VARIANT	12	12	G -> A (in a colorectal cancer sample;
FT				somatic mutation).
FT				/FTId=VAR_036305.
FT	VARIANT	12	12	G -> C (in lung carcinoma; somatic
FT				mutation).
FT				/FTId=VAR_006839.
FT	VARIANT	12	12	G -> D (in pancreatic carcinoma, GASC and
FT				lung carcinoma; somatic mutation).
FT				/FTId=VAR_016026.
FT	VARIANT	12	12	G -> R (in lung cancer and bladder
FT				cancer; somatic mutation).
FT				/FTId=VAR_016027.
FT	VARIANT	12	12	G -> S (in lung carcinoma and GASC;
FT				somatic mutation).
FT				/FTId=VAR_016028.
FT	VARIANT	12	12	G -> V (in lung carcinoma, pancreatic
FT				carcinoma, colon cancer and GASC; somatic
FT				mutation).
FT				/FTId=VAR_006840.
FT	VARIANT	13	13	G -> D (in a breast carcinoma cell line
FT				and GASC; somatic mutation).
FT				/FTId=VAR_016029.
FT	VARIANT	13	13	G -> R (in pylocytic astrocytoma; somatic
FT				mutation; increase activation of the Ras
FT				pathway).
FT				/FTId=VAR_065145.
FT	VARIANT	14	14	V -> I (in NS3; affects activity and
FT				impairs responsiveness to GTPase
FT				activating proteins; characterized by a
FT				strong increase of both intrinsic and
FT				guanine nucleotide exchanged factor-
FT				catalyzed nucleotide exchange leading to
FT				an increased level of the activated
FT				state).
FT				/FTId=VAR_026109.
FT	VARIANT	22	22	Q -> E (in CFC syndrome; exhibits an
FT				increase in intrinsic and guanine
FT				nucleotide exchange factor catalyzed
FT				nucleotide exchange in combination with
FT				an impaired GTPase-activating protein-
FT				stimulated GTP hydrolysis but functional
FT				in interaction with effectors).

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FT				/FTId=VAR_064850.
FT	VARIANT	22	22	Q -> R (in NS3; impairs GTPase-activating
FT				protein stimulated GTP hydrolysis with
FT				unaffected intrinsic functions and a
FT				virtually functional effector
FT				interaction).
FT				/FTId=VAR_064851.
FT	VARIANT	34	34	P -> L (in NS3; characterized by a
FT				defective GTPase-activating protein
FT				sensitivity and a strongly reduced
FT				interaction with effectors).
FT				/FTId=VAR_064852.
FT	VARIANT	34	34	P -> Q (in NS3).
FT				/FTId=VAR_064853.
FT	VARIANT	34	34	P -> R (in CFC syndrome; characterized by
FT				a defective GTPase-activating protein
FT				sensitivity and a strongly reduced
FT				interaction with effectors).
FT				/FTId=VAR_026110.
FT	VARIANT	36	36	I -> M (in NS3).
FT				/FTId=VAR_064854.
FT	VARIANT	58	58	T -> I (in NS3; affects activity and
FT				impairs responsiveness to GTPase
FT				activating proteins; exhibits only minor
FT				alterations in its in vitro biochemical
FT				behavior compared to wild-type protein).
FT				/FTId=VAR_026111.
FT	VARIANT	59	59	A -> T (in bladder cancer and GASC;
FT				somatic mutation).
FT				/FTId=VAR_016030.
FT	VARIANT	60	60	G -> R (in CFC syndrome; characterized by
FT				a defective GTPase-activating protein
FT				sensitivity and a strongly reduced
FT				interaction with effectors).
FT				/FTId=VAR_026112.
FT	VARIANT	60	60	G -> S (in NS3).
FT				/FTId=VAR_065146.
FT	VARIANT	61	61	Q -> H (in lung carcinoma;
FT				dbSNP:rs17851045).
FT				/FTId=VAR_006841.
FT	VARIANT	61	61	Q -> R (in a colorectal cancer sample;
FT				somatic mutation).
FT				/FTId=VAR_036306.
FT	VARIANT	117	117	K -> N (in a colorectal cancer sample;
FT				somatic mutation).
FT				/FTId=VAR_036307.
FT	VARIANT	146	146	A -> T (in a colorectal cancer sample;
FT				somatic mutation).
FT				/FTId=VAR_036308.
FT	MUTAGEN	164	164	R->A: Loss of GTP-binding activity.
FT	STRAND	2	9	
FT	HELIX	16	25	
FT	STRAND	36	46	
FT	STRAND	49	58	
FT	HELIX	68	74	
FT	STRAND	76	83	
FT	HELIX	87	103	
FT	STRAND	111	116	
FT	STRAND	120	122	
FT	HELIX	127	137	
FT	STRAND	141	143	

FT	TURN	146	148
FT	HELIX	152	164

SEQ ID No. 23: Amino Acid Sequence of Mutant KRAS (G12C KRAS)**3. Sequences relating to HRAS****3.1 NM_001130442.1 → NP_001123914.1 GTPase HRas isoform 1:****Description**

Transcript Variant: This variant (3) differs in the 3' UTR, compared to variant 1. Variants 1 and 3 encode the same protein.

SEQ ID No. 24: Nucleotide Sequence (570 nt) of HRAS, Isoform 1, Transcript Variant (3)**SEQ ID No. 25: Amino Acid Sequence (189 aa) of HRAS, Isoform 1, Transcript Variant (3)****3.2 NM_005343.2 → NP_005334.1 GTPase HRas isoform 1:****Description**

Transcript Variant: This variant (1) encodes the longer isoform (1). Variants 1 and 3 encode the same protein.

SEQ ID No. 26: Nucleotide Sequence (570 nt) of HRAS, Isoform 1, Transcript Variant (1)**SEQ ID No. 27: Amino Acid Sequence (189 aa) of HRAS, Isoform 1, Transcript Variant (1)****3.3 NM_176795.3 → NP_789765.1 GTPase HRas isoform 2:****Description**

Transcript Variant: This variant (2) differs in the 3' UTR and includes an alternate exon in its 3' coding region, compared to variant 1. The resulting isoform (2) contains a distinct C-terminus and is shorter than isoform 1.

SEQ ID No. 28: Nucleotide Sequence (513 nt) of HRAS, Isoform 2, Transcript Variant (2)**SEQ ID No. 29: Amino Acid Sequence (170 aa) of HRAS, Isoform 2, Transcript Variant (2)****HRAS Sequence Annotation**

In this Sequence Annotation, common mutations of HRAS are indicated by the term “variant”.

FT	CHAIN	1	186	GTPase HRas.
FT				/FTId=PRO_0000042996.
FT	INIT_MET	1	1	Removed; alternate.
FT	CHAIN	2	186	GTPase HRas, N-terminally processed.
FT				/FTId=PRO_0000326476.
FT	PROPEP	187	189	Removed in mature form.
FT				/FTId=PRO_0000042997.
FT	NP_BIND	10	17	GTP.
FT	NP_BIND	57	61	GTP.
FT	NP_BIND	116	119	GTP.
FT	REGION	166	185	Hypervariable region.
FT	MOTIF	32	40	Effector region.
FT	MOD_RES	1	1	N-acetylmethionine; in GTPase HRas;
FT				alternate.
FT	MOD_RES	2	2	N-acetylthreonine; in GTPase HRas, N-
FT				terminally processed.
FT	MOD_RES	118	118	S-nitrosocysteine.
FT	MOD_RES	186	186	Cysteine methyl ester.
FT	LIPID	181	181	S-palmitoyl cysteine.
FT	LIPID	184	184	S-(15-deoxy-Delta12,14-prostaglandin J2-
FT				9-yl)cysteine; alternate.
FT	LIPID	184	184	S-palmitoyl cysteine; alternate.
FT	LIPID	186	186	S-farnesyl cysteine.
FT	VAR_SEQ	152	189	VEDAFYTLVREIRQHKLRKLNPPDESGPGCMSCKCVLS ->
FT				SRSGSSSSSGTLWDPPGPM (in isoform 2).
FT				/FTId=VSP_041597.
FT	VARIANT	12	12	G -> A (in FCSS).
FT				/FTId=VAR_026106.
FT	VARIANT	12	12	G -> C (in FCSS).
FT				/FTId=VAR_045975.
FT	VARIANT	12	12	G -> E (in FCSS).
FT				/FTId=VAR_045976.
FT	VARIANT	12	12	G -> S (in FCSS, OSCC and CMEMS).
FT				/FTId=VAR_006837.
FT	VARIANT	12	12	G -> V (in FCSS, bladder carcinoma and
FT				CMEMS; constitutively activated;
FT				interacts and recruits PLCE1 to plasma
FT				membrane; loss of interaction with and
FT				recruitment to plasma membrane of PLCE1
FT				when associated with F-32; loss of
FT				interaction with PLCE1 when associated
FT				with G-26, F-32 and S-35; no effect on
FT				interaction with PLCE1 when associated
FT				with A-29, G-34, G-37, N-38 and C-39; no
FT				effect on subcellular location of isoform
FT				2).
FT				/FTId=VAR_006836.
FT	VARIANT	13	13	G -> C (in FCSS).
FT				/FTId=VAR_026107.
FT	VARIANT	13	13	G -> D (in FCSS).
FT				/FTId=VAR_026108.
FT	VARIANT	22	22	Q -> K (in CMEMS).
FT				/FTId=VAR_045977.
FT	VARIANT	58	58	T -> I (in FCSS).
FT				/FTId=VAR_045978.
FT	VARIANT	61	61	Q -> K (in follicular thyroid carcinoma
FT				samples; somatic mutation; increases
FT				transformation of cultured cell lines;
FT				dbSNP:rs28933406).

FT				/FTId=VAR_045979.
FT	VARIANT	61	61	Q -> L (in melanoma; strongly reduced GTP hydrolysis in the presence of RAF1; increases transformation of cultured cell lines).
FT				/FTId=VAR_006838.
FT	VARIANT	63	63	E -> K (in CMEMS).
FT				/FTId=VAR_045980.
FT	VARIANT	117	117	K -> R (in FCSS).
FT				/FTId=VAR_045981.
FT	VARIANT	146	146	A -> T (in FCSS).
FT				/FTId=VAR_045982.
FT	VARIANT	146	146	A -> V (in FCSS).
FT				/FTId=VAR_045983.
FT	MUTAGEN	17	17	S->N: Dominant negative. Prevents PLCE1 EGF-induced recruitment to plasma membrane. No effect on subcellular location of isoform 2.
FT				
FT	MUTAGEN	26	26	N->G: Loss of interaction with PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	29	29	V->A: No effect on interaction with PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	32	32	Y->F: Loss of interaction and recruitment to plasma membrane of PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	34	34	P->G: No effect on interaction with PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	35	35	T->S: Loss of interaction with PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	37	37	E->G: No effect on interaction with PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	38	38	D->N: No effect on interaction with PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	39	39	S->C: No effect on interaction with PLCE1; when associated with V-12.
FT				
FT	MUTAGEN	59	59	A->T: Loss of GTPase activity and creation of an autophosphorylation site.
FT				
FT	MUTAGEN	61	61	Q->I: Moderately increased transformation of cultured cell lines.
FT				
FT	MUTAGEN	61	61	Q->V: Strongly increased transformation of cultured cell lines.
FT				
FT	MUTAGEN	83	83	A->T: GTP-binding activity reduced by factor of 30.
FT				
FT	MUTAGEN	118	118	C->S: Abolishes S-nitrosylation. No stimulation of guanine nucleotide exchange.
FT				
FT	MUTAGEN	119	119	D->N: Loss of GTP-binding activity.
FT				
FT	MUTAGEN	144	144	T->I: GTP-binding activity reduced by factor of 25.
FT				
FT	MUTAGEN	164	165	RQ->AV: Loss of GTP-binding activity.
FT				
FT	MUTAGEN	181	181	C->S: Exclusively localized in Golgi. Non-specifically localized on all endomembranes; when associated with S-184.
FT				
FT	MUTAGEN	184	184	C->S: Loss of S-(15-deoxy-Delta12,14-prostaglandin J2-9-yl)cysteine stimulation of Ras-GTPase activity. Mainly localized in Golgi. Non-specifically localized on all endomembranes; when associated with S-181.
FT				

FT	STRAND	3	11
FT	STRAND	12	14
FT	HELIX	16	25
FT	STRAND	27	31
FT	STRAND	38	46
FT	STRAND	49	57
FT	STRAND	60	63
FT	HELIX	66	74
FT	STRAND	76	83
FT	TURN	84	86
FT	HELIX	87	104
FT	STRAND	105	107
FT	STRAND	111	116
FT	STRAND	120	122
FT	HELIX	127	136
FT	STRAND	141	144
FT	TURN	146	148
FT	HELIX	152	164

Sequences relating to NRAS

3.4 NM_002524.4 → NP_002515.1 GTPase NRas:

SEQ ID No. 30: Nucleotide Sequence (570 nt) of NRAS

SEQ ID No. 31: Amino Acid Sequence (189 aa) of NRAS

NRAS Sequence Annotation

In this Sequence Annotation, common mutations of NRAS are indicated by the term "variant".

FT	CHAIN	1	186	GTPase NRas.
FT				/FTId=PRO_0000043006.
FT	PROPEP	187	189	Removed in mature form (By similarity).
FT				/FTId=PRO_0000043007.
FT	NP_BIND	10	17	GTP.
FT	NP_BIND	57	61	GTP.
FT	NP_BIND	116	119	GTP.
FT	REGION	166	185	Hypervariable region.
FT	MOTIF	32	40	Effector region.
FT	MOD_RES	2	2	Phosphothreonine.
FT	MOD_RES	4	4	Phosphotyrosine.
FT	MOD_RES	186	186	Cysteine methyl ester (By similarity).
FT	LIPID	181	181	S-palmitoyl cysteine.
FT	LIPID	186	186	S-farnesyl cysteine.
FT	VARIANT	12	12	G -> C (in leukemia).
FT				/FTId=VAR_021194.
FT	VARIANT	13	13	G -> D (in ALPS4).
FT				/FTId=VAR_063084.
FT	VARIANT	13	13	G -> R (in colorectal cancer).
FT				/FTId=VAR_006845.
FT	VARIANT	50	50	T -> I (in NS6; hypermorphic mutation).
FT				/FTId=VAR_063085.
FT	VARIANT	60	60	G -> E (in NS6; hypermorphic mutation).
FT				/FTId=VAR_063086.

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FT	VARIANT	61	61	Q -> K (in neuroblastoma cell).
FT				/FTId=VAR_006846.
FT	VARIANT	61	61	Q -> R (in lung carcinoma cell and
FT				melanoma; dbSNP:rs11554290).
FT				/FTId=VAR_006847.
FT	MUTAGEN	164	164	R->A: Loss of GTP-binding activity.
FT	STRAND	2	9	
FT	HELIX	16	25	
FT	STRAND	38	46	
FT	STRAND	49	57	
FT	STRAND	76	83	
FT	HELIX	87	104	
FT	STRAND	111	116	
FT	HELIX	127	137	
FT	STRAND	141	143	
FT	TURN	146	148	
FT	HELIX	152	166	

4. Sequences relating to ALK

4.1 NM_004304.4 → NP_004295.2 ALK tyrosine kinase receptor precursor:

SEQ ID No. 32: Nucleotide Sequence (4863 nt) of ALK tyrosine kinase receptor precursor

SEQ ID No. 33: Amino Acid Sequence (1620 aa) of ALK tyrosine kinase receptor precursor

Aberrations of ALK

CC -!- DISEASE: Note=A chromosomal aberration involving ALK is found in a
 CC form of non-Hodgkin lymphoma. Translocation t(2;5)(p23;q35) with
 CC NPM1. The resulting chimeric NPM1-ALK protein homodimerize and the
 CC kinase becomes constitutively activated. The constitutively active
 CC fusion proteins are responsible for 5-10% of non-Hodgkin
 CC lymphomas.
 CC -!- DISEASE: Note=A chromosomal aberration involving ALK is associated
 CC with inflammatory myofibroblastic tumors (IMTs). Translocation
 CC t(2;11)(p23;p15) with CARS; translocation t(2;4)(p23;q21) with
 CC SEC31A.
 CC -!- DISEASE: Note=A chromosomal aberration involving ALK is associated
 CC with anaplastic large-cell lymphoma (ALCL). Translocation
 CC t(2;17)(p23;q25) with ALO17.
 CC -!- DISEASE: Defects in ALK are the cause of susceptibility to
 CC neuroblastoma type 3 (NBLST3) [MIM:613014]. Neuroblastoma is a
 CC common neoplasm of early childhood arising from embryonic cells
 CC that form the primitive neural crest and give rise to the adrenal
 CC medulla and the sympathetic nervous system.
 CC -!- DISEASE: Note=The ALK signaling pathway plays an important role in
 CC glioblastoma, the most common malignant brain tumor of adults and
 CC one of the most lethal cancers. It regulates both glioblastoma
 CC migration and growth.

ALK Sequence annotation

In this Sequence Annotation, common mutations of ALK are indicated by the term “variant”.

FT	SIGNAL	1	18	Potential.
FT	CHAIN	19	1620	ALK tyrosine kinase receptor.
FT				/FTId=PRO_0000016740.
FT	TOPO_DOM	19	1038	Extracellular (Potential).
FT	TRANSMEM	1039	1059	Helical; (Potential).
FT	TOPO_DOM	1060	1620	Cytoplasmic (Potential).
FT	DOMAIN	264	427	MAM 1.
FT	DOMAIN	437	473	LDL-receptor class A.
FT	DOMAIN	478	636	MAM 2.
FT	DOMAIN	1116	1392	Protein kinase.
FT	NP_BIND	1197	1199	ATP.
FT	REGION	1197	1199	Inhibitor binding.
FT	COMPBIAS	816	940	Gly-rich.
FT	ACT_SITE	1249	1249	Proton acceptor (By similarity).
FT	BINDING	1124	1124	ATP; via carbonyl oxygen.
FT	BINDING	1150	1150	ATP (By similarity).
FT	BINDING	1150	1150	Inhibitor.
FT	BINDING	1199	1199	Inhibitor; via amide nitrogen.
FT	BINDING	1203	1203	Inhibitor.
FT	BINDING	1210	1210	Inhibitor.
FT	BINDING	1270	1270	ATP.
FT	MOD_RES	211	211	Phosphoserine.
FT	MOD_RES	1078	1078	Phosphotyrosine.
FT	MOD_RES	1092	1092	Phosphotyrosine.
FT	MOD_RES	1096	1096	Phosphotyrosine.
FT	MOD_RES	1131	1131	Phosphotyrosine.
FT	MOD_RES	1278	1278	Phosphotyrosine.
FT	MOD_RES	1282	1282	Phosphotyrosine; by autocatalysis.
FT	MOD_RES	1283	1283	Phosphotyrosine.
FT	MOD_RES	1359	1359	Phosphotyrosine.
FT	MOD_RES	1507	1507	Phosphotyrosine.
FT	MOD_RES	1584	1584	Phosphotyrosine.
FT	MOD_RES	1604	1604	Phosphotyrosine.
FT	CARBOHYD	169	169	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	244	244	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	285	285	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	324	324	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	411	411	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	424	424	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	445	445	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	563	563	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	571	571	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	627	627	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	709	709	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	808	808	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	863	863	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	864	864	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	886	886	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	986	986	N-linked (GlcNAc...) (Potential).
FT	VARIANT	90	90	S -> L (in dbSNP:rs34617074).
FT				/FTId=VAR_041477.
FT	VARIANT	163	163	V -> L (in dbSNP:rs55697431).
FT				/FTId=VAR_041478.
FT	VARIANT	296	296	E -> Q (in dbSNP:rs56077855).
FT				/FTId=VAR_041479.
FT	VARIANT	476	476	V -> A (in dbSNP:rs35093491).
FT				/FTId=VAR_041480.
FT	VARIANT	560	560	L -> F (in a breast pleomorphic lobular

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FT				carcinoma sample; somatic mutation).
FT				/FTid=VAR_041481.
FT	VARIANT	680	680	T -> I (in dbSNP:rs35228363).
FT				/FTid=VAR_041482.
FT	VARIANT	704	704	A -> T (in dbSNP:rs34829159).
FT				/FTid=VAR_041483.
FT	VARIANT	868	868	L -> Q (in dbSNP:rs55941323).
FT				/FTid=VAR_061288.
FT	VARIANT	877	877	A -> S (in an ovarian serous carcinoma
FT				sample; somatic mutation).
FT				/FTid=VAR_041484.
FT	VARIANT	1012	1012	T -> M (in dbSNP:rs35073634).
FT				/FTid=VAR_041485.
FT	VARIANT	1091	1091	D -> N (in NBLST3; somatic mutation).
FT				/FTid=VAR_063850.
FT	VARIANT	1121	1121	G -> D (in dbSNP:rs55760835).
FT				/FTid=VAR_041486.
FT	VARIANT	1128	1128	G -> A (in NBLST3).
FT				/FTid=VAR_063851.
FT	VARIANT	1151	1151	T -> M (in NBLST3).
FT				/FTid=VAR_063852.
FT	VARIANT	1166	1166	M -> R (in NBLST3; somatic mutation).
FT				/FTid=VAR_063853.
FT	VARIANT	1171	1171	I -> N (in NBLST3; somatic mutation).
FT				/FTid=VAR_063854.
FT	VARIANT	1174	1174	F -> C (in NBLST3).
FT				/FTid=VAR_063855.
FT	VARIANT	1174	1174	F -> I (in NBLST3; somatic mutation).
FT				/FTid=VAR_063856.
FT	VARIANT	1174	1174	F -> L (in NBLST3; somatic mutation).
FT				/FTid=VAR_063857.
FT	VARIANT	1174	1174	F -> V (in NBLST3; somatic mutation).
FT				/FTid=VAR_063858.
FT	VARIANT	1192	1192	R -> P (in NBLST3).
FT				/FTid=VAR_063859.
FT	VARIANT	1234	1234	A -> T (in NBLST3; somatic mutation).
FT				/FTid=VAR_063860.
FT	VARIANT	1245	1245	F -> C (in NBLST3; somatic mutation).
FT				/FTid=VAR_063861.
FT	VARIANT	1245	1245	F -> V (in NBLST3; somatic mutation).
FT				/FTid=VAR_063862.
FT	VARIANT	1250	1250	I -> T (in NBLST3; somatic mutation).
FT				/FTid=VAR_063863.
FT	VARIANT	1274	1274	A -> T (in dbSNP:rs45502292).
FT				/FTid=VAR_041487.
FT	VARIANT	1275	1275	R -> L (observed in neuroblastoma).
FT				/FTid=VAR_063864.
FT	VARIANT	1275	1275	R -> Q (in NBLST3).
FT				/FTid=VAR_063865.
FT	VARIANT	1278	1278	Y -> S (in NBLST3; somatic mutation).
FT				/FTid=VAR_063866.
FT	VARIANT	1328	1328	M -> L (in dbSNP:rs56160491).
FT				/FTid=VAR_041488.
FT	VARIANT	1376	1376	F -> S (in dbSNP:rs17694720).
FT				/FTid=VAR_055987.
FT	VARIANT	1416	1416	K -> N (in dbSNP:rs55782189).
FT				/FTid=VAR_041489.
FT	VARIANT	1419	1419	E -> K (in dbSNP:rs56181542).
FT				/FTid=VAR_041490.
FT	VARIANT	1429	1429	Q -> R (in dbSNP:rs55906201).
FT				/FTid=VAR_041491.

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FT	VARIANT	1461	1461	I -> V (in dbSNP:rs1670283).
FT				/FTId=VAR_031042.
FT	VARIANT	1491	1491	K -> R (in dbSNP:rs1881420).
FT				/FTId=VAR_031043.
FT	VARIANT	1529	1529	D -> E (in dbSNP:rs1881421).
FT				/FTId=VAR_031044.
FT	VARIANT	1599	1599	P -> H (in dbSNP:rs1881423).
FT				/FTId=VAR_055988.
FT	MUTAGEN	1507	1507	Y->F: Impairs interaction with SHC1.
FT	CONFLICT	36	36	P -> S (in Ref. 1; AAB71619).
FT	HELIX	1087	1092	
FT	STRAND	1096	1098	
FT	STRAND	1101	1103	
FT	HELIX	1105	1107	
FT	HELIX	1113	1115	
FT	STRAND	1117	1121	
FT	STRAND	1126	1128	
FT	STRAND	1130	1134	
FT	STRAND	1137	1140	
FT	STRAND	1146	1151	
FT	HELIX	1158	1173	
FT	STRAND	1182	1186	
FT	STRAND	1188	1191	
FT	STRAND	1193	1197	
FT	HELIX	1204	1210	
FT	STRAND	1215	1217	
FT	HELIX	1223	1242	
FT	HELIX	1252	1254	
FT	STRAND	1255	1258	
FT	STRAND	1260	1263	
FT	STRAND	1266	1268	
FT	HELIX	1272	1278	
FT	STRAND	1280	1282	
FT	HELIX	1288	1290	
FT	HELIX	1293	1295	
FT	HELIX	1298	1303	
FT	HELIX	1308	1323	
FT	HELIX	1335	1343	
FT	HELIX	1356	1365	
FT	HELIX	1370	1372	
FT	HELIX	1376	1388	
FT	HELIX	1390	1393	
FT	STRAND	1574	1576	
FT	STRAND	1582	1584	

SEQ ID No. 34: Nucleotide Sequence of EML4-ALK

>ENA|EU236948|EU236948.1 Homo sapiens EML4/ALK fusion protein variant 3 (EML4/ALK fusion) mRNA, complete cds. : Location:1..2391

SEQ ID No. 35: Amino Acid Sequence of EML4-ALK**SEQ ID No. 36: Nucleotide Sequence of EML4-ALK variant 6**

>ENA|AB462411|AB462411.1 Homo sapiens EML4-ALK variant 6 mRNA for fusion protein EML4-ALK variant 6, complete cds. : Location:1..3365

SEQ ID No. 37: Amino Acid Sequence of EML4-ALK variant 6**SEQ ID No. 38: Nucleotide Sequence of EML4-ALK variant 7**

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>ENA|AB462412|AB462412.1 Homo sapiens EML4-ALK variant 7 mRNA for fusion protein EML4-ALK variant 7, complete cds. : Location:1..3435

SEQ ID No. 39: Amino Acid Sequence of EML4-ALK variant 7

SEQ ID No. 40: Nucleotide Sequence of EML4-ALK fusion protein variant 8a

>ENA|GU797894|GU797894.1 Homo sapiens EML4-ALK fusion protein variant 8a mRNA, partial cds. : Location:1..171

SEQ ID No. 41: Amino Acid Sequence of EML4-ALK fusion protein variant 8a

SEQ ID No. 42: EML4-ALK fusion protein variant 8b

>ENA|GU797895|GU797895.1 Homo sapiens EML4-ALK fusion protein variant 8b mRNA, partial cds. : Location:1..236

SEQ ID No. 43: Amino Acid Sequence of EML4-ALK fusion protein variant 8b

5. Sequences relating to MET (also known as c-Met)

5.1 NM_000245.2 → NP_000236.2 hepatocyte growth factor receptor isoform b precursor:

Description

Transcript Variant: This variant (2) uses an alternate in-frame splice junction at the end of an exon compared to variant 1. The resulting isoform (b) has the same N- and C-termini but is shorter compared to isoform a.

SEQ ID No. 44: Nucleotide Sequence (4173 nt) of MET, hepatocyte growth factor receptor isoform b precursor, Transcript Variant (2)

SEQ ID No. 45: Amino Acid Sequence (1390 aa) of MET, hepatocyte growth factor receptor isoform b precursor, Transcript Variant (2)

5.2 NM_001127500.1 → NP_001120972.1 hepatocyte growth factor receptor isoform a precursor:

Description

Transcript Variant: This variant (1) represents the longer transcript and encodes the longer isoform (a).

SEQ ID No. 46: Nucleotide Sequence (4227 nt) of MET, hepatocyte growth factor receptor isoform a precursor, Transcript Variant (1)

SEQ ID No. 47: Amino Acid Sequence (1408 aa) of MET, hepatocyte growth factor receptor isoform a precursor, Transcript Variant (1)

Aberrations of MET

CC -!- DISEASE: Note=Activation of MET after rearrangement with the TPR

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CC gene produces an oncogenic protein.

CC -!- DISEASE: Note=Defects in MET may be associated with gastric

CC cancer.

CC -!- DISEASE: Defects in MET are a cause of hepatocellular carcinoma

CC (HCC) [MIM:114550].

CC -!- DISEASE: Defects in MET are a cause of renal cell carcinoma

CC papillary (RCCP) [MIM:605074]. It is a subtype of renal cell

CC carcinoma tending to show a tubulo-papillary architecture formed

CC by numerous, irregular, finger-like projections of connective

CC tissue. Renal cell carcinoma is a heterogeneous group of sporadic

CC or hereditary carcinoma derived from cells of the proximal renal

CC tubular epithelium. It is subclassified into common renal cell

CC carcinoma (clear cell, non-papillary carcinoma), papillary renal

CC cell carcinoma, chromophobe renal cell carcinoma, collecting duct

CC carcinoma with medullary carcinoma of the kidney, and unclassified

CC renal cell carcinoma.

CC -!- DISEASE: Note=A common allele in the promoter region of the MET

CC shows genetic association with susceptibility to autism in some

CC families. Functional assays indicate a decrease in MET promoter

CC activity and altered binding of specific transcription factor

CC complexes.

CC -!- DISEASE: Note=MET activating mutations may be involved in the

CC development of a highly malignant, metastatic syndrome known as

CC cancer of unknown primary origin (CUP) or primary occult

CC malignancy. Systemic neoplastic spread is generally a late event

CC in cancer progression. However, in some instances, distant

CC dissemination arises at a very early stage, so that metastases

CC reach clinical relevance before primary lesions. Sometimes, the

CC primary lesions cannot be identified in spite of the progresses in

CC the diagnosis of malignancies.

MET Sequence Annotation

In this Sequence Annotation, common mutations of MET are indicated by the term "variant".

FT	SIGNAL	1	24	Potential.
FT	CHAIN	25	1390	Hepatocyte growth factor receptor.
FT				/FTId=PRO_0000024440.
FT	TOPO_DOM	25	932	Extracellular (Potential).
FT	TRANSMEM	933	955	Helical; (Potential).
FT	TOPO_DOM	956	1390	Cytoplasmic (Potential).
FT	DOMAIN	27	515	Sema.
FT	DOMAIN	563	655	IPT/TIG 1.
FT	DOMAIN	657	739	IPT/TIG 2.
FT	DOMAIN	742	836	IPT/TIG 3.
FT	DOMAIN	1078	1345	Protein kinase.
FT	NP_BIND	1084	1092	ATP (By similarity).
FT	REGION	1212	1390	Interaction with RANBP9.
FT	REGION	1320	1359	Interaction with MUC20.
FT	ACT_SITE	1204	1204	Proton acceptor (By similarity).
FT	BINDING	1110	1110	ATP.
FT	SITE	307	308	Cleavage (Potential).
FT	SITE	1009	1010	Breakpoint for translocation to form TPR-
FT				MET oncogene.
FT	MOD_RES	966	966	Phosphoserine.
FT	MOD_RES	977	977	Phosphothreonine.
FT	MOD_RES	988	988	Phosphoserine.
FT	MOD_RES	990	990	Phosphoserine.
FT	MOD_RES	997	997	Phosphoserine.
FT	MOD_RES	1000	1000	Phosphoserine.

FT	MOD_RES	1003	1003	Phosphotyrosine.
FT	MOD_RES	1230	1230	Phosphotyrosine.
FT	MOD_RES	1234	1234	Phosphotyrosine; by autocatalysis.
FT	MOD_RES	1235	1235	Phosphotyrosine; by autocatalysis.
FT	MOD_RES	1289	1289	Phosphothreonine.
FT	MOD_RES	1349	1349	Phosphotyrosine; by autocatalysis.
FT	MOD_RES	1356	1356	Phosphotyrosine; by autocatalysis.
FT	MOD_RES	1365	1365	Phosphotyrosine.
FT	CARBOHYD	45	45	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	106	106	N-linked (GlcNAc...).
FT	CARBOHYD	149	149	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	202	202	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	399	399	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	405	405	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	607	607	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	635	635	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	785	785	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	879	879	N-linked (GlcNAc...) (Potential).
FT	CARBOHYD	930	930	N-linked (GlcNAc...) (Potential).
FT	DISULFID	95	101	
FT	DISULFID	98	160	
FT	DISULFID	133	141	
FT	DISULFID	172	175	
FT	DISULFID	298	363	
FT	DISULFID	385	397	
FT	DISULFID	520	538	
FT	DISULFID	526	561	
FT	DISULFID	529	545	
FT	DISULFID	541	551	
FT	VAR_SEQ	755	764	SGGSTITGVG -> RHNIALIQR (in isoform 3).
FT				/FTId=VSP_042447.
FT	VAR_SEQ	755	755	S -> STWWKEPLNIVSFLFCFAS (in isoform 2).
FT				/FTId=VSP_005005.
FT	VAR_SEQ	765	1390	Missing (in isoform 3).
FT				/FTId=VSP_042448.
FT	VARIANT	143	143	R -> Q (in dbSNP:rs35469582).
FT				/FTId=VAR_041738.
FT	VARIANT	150	150	H -> Y (found in a case of cancer of
FT				unknown primary origin; the mutated
FT				receptor is still functional and can
FT				sustain the transformed phenotype;
FT				somatic mutation).
FT				/FTId=VAR_064855.
FT	VARIANT	156	156	S -> L (in dbSNP:rs56311081).
FT				/FTId=VAR_041739.
FT	VARIANT	168	168	E -> D (found in a case of cancer of
FT				unknown primary origin; the mutated
FT				receptor is still functional and can
FT				sustain the transformed phenotype;
FT				somatic mutation; dbSNP:rs55985569).
FT				/FTId=VAR_041740.
FT	VARIANT	238	238	L -> S (in dbSNP:rs34349517).
FT				/FTId=VAR_032478.
FT	VARIANT	316	316	I -> M (in dbSNP:rs35225896).
FT				/FTId=VAR_032479.
FT	VARIANT	320	320	A -> V (in dbSNP:rs35776110).
FT				/FTId=VAR_006285.
FT	VARIANT	375	375	N -> S (in dbSNP:rs33917957).
FT				/FTId=VAR_032480.
FT	VARIANT	385	385	C -> Y (found in a case of cancer of
FT				unknown primary origin; the mutated

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FT				receptor is still functional and can
FT				sustain the transformed phenotype;
FT				somatic mutation).
FT				/FTId=VAR_064856.
FT	VARIANT	773	773	P -> L (in gastric cancer).
FT				/FTId=VAR_032481.
FT	VARIANT	970	970	R -> C (in dbSNP:rs34589476).
FT				/FTId=VAR_032482.
FT	VARIANT	991	991	P -> S (in gastric cancer; prolonged
FT				tyrosine phosphorylation in response to
FT				HGF/SF; transforming activity in athymic
FT				nude mice).
FT				/FTId=VAR_032483.
FT	VARIANT	992	992	T -> I (found in a case of cancer of
FT				unknown primary origin; the mutated
FT				receptor is still functional and can
FT				sustain the transformed phenotype;
FT				somatic mutation; dbSNP:rs56391007).
FT				/FTId=VAR_032484.
FT	VARIANT	1092	1092	V -> I (in RCCP; constitutive
FT				autophosphorylation).
FT				/FTId=VAR_032485.
FT	VARIANT	1094	1094	H -> L (in RCCP; constitutive
FT				autophosphorylation; causes malignant
FT				transformation in cell lines).
FT				/FTId=VAR_032486.
FT	VARIANT	1094	1094	H -> R (in RCCP; causes malignant
FT				transformation in cell lines).
FT				/FTId=VAR_032487.
FT	VARIANT	1094	1094	H -> Y (in RCCP; constitutive
FT				autophosphorylation; causes malignant
FT				transformation in cell lines).
FT				/FTId=VAR_032488.
FT	VARIANT	1106	1106	H -> D (in RCCP; constitutive
FT				autophosphorylation; causes malignant
FT				transformation in cell lines).
FT				/FTId=VAR_032489.
FT	VARIANT	1131	1131	M -> T (in RCCP; germline mutation).
FT				/FTId=VAR_006286.
FT	VARIANT	1173	1173	T -> I (in HCC).
FT				/FTId=VAR_032490.
FT	VARIANT	1188	1188	V -> L (in RCCP; germline mutation).
FT				/FTId=VAR_006287.
FT	VARIANT	1195	1195	L -> V (in RCCP; somatic mutation).
FT				/FTId=VAR_006288.
FT	VARIANT	1220	1220	V -> I (in RCCP; germline mutation).
FT				/FTId=VAR_006289.
FT	VARIANT	1228	1228	D -> H (in RCCP; somatic mutation).
FT				/FTId=VAR_006291.
FT	VARIANT	1228	1228	D -> N (in RCCP; germline mutation).
FT				/FTId=VAR_006290.
FT	VARIANT	1230	1230	Y -> C (in RCCP; germline mutation).
FT				/FTId=VAR_006292.
FT	VARIANT	1230	1230	Y -> D (in RCCP; constitutive
FT				autophosphorylation; causes malignant
FT				transformation in cell lines).
FT				/FTId=VAR_032491.
FT	VARIANT	1230	1230	Y -> H (in RCCP; somatic mutation).
FT				/FTId=VAR_006293.
FT	VARIANT	1244	1244	K -> R (in HCC).
FT				/FTId=VAR_032492.

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FT	VARIANT	1250	1250	M -> I (in HCC).
FT				/FTId=VAR_032493.
FT	VARIANT	1250	1250	M -> T (in RCCP; somatic mutation).
FT				/FTId=VAR_006294.
FT	VARIANT	1294	1294	V -> I (found in a case of cancer of
FT				unknown primary origin; the mutated
FT				receptor is still functional and can
FT				sustain the transformed phenotype;
FT				somatic mutation).
FT				/FTId=VAR_064857.
FT	CONFLICT	237	237	V -> A (in Ref. 3; ACF47606).
FT	CONFLICT	508	508	K -> R (in Ref. 3; ACF47606).
FT	CONFLICT	720	720	F -> S (in Ref. 3; ACF47606).
FT	CONFLICT	1191	1191	G -> A (in Ref. 1; AAA59591).
FT	CONFLICT	1272	1272	L -> V (in Ref. 1; AAA59591, 2; CAB56793
FT				and 6; AAA59590).
FT	STRAND	45	47	
FT	STRAND	52	58	
FT	STRAND	61	66	
FT	STRAND	69	74	
FT	TURN	75	77	
FT	STRAND	80	84	
FT	STRAND	89	91	
FT	STRAND	93	95	
FT	STRAND	97	99	
FT	STRAND	102	104	
FT	STRAND	111	113	
FT	STRAND	119	123	
FT	STRAND	125	133	
FT	STRAND	135	139	
FT	STRAND	141	145	
FT	STRAND	154	160	
FT	STRAND	182	189	
FT	STRAND	192	199	
FT	STRAND	213	219	
FT	HELIX	231	233	
FT	HELIX	239	241	
FT	TURN	242	244	
FT	STRAND	247	255	
FT	STRAND	258	268	
FT	STRAND	272	274	
FT	STRAND	277	281	
FT	STRAND	284	286	
FT	STRAND	292	300	
FT	STRAND	312	314	
FT	STRAND	316	323	
FT	HELIX	327	333	
FT	STRAND	341	349	
FT	STRAND	356	366	
FT	HELIX	367	374	
FT	HELIX	387	390	
FT	STRAND	392	394	
FT	TURN	395	398	
FT	STRAND	418	422	
FT	STRAND	424	427	
FT	TURN	429	436	
FT	STRAND	439	447	
FT	STRAND	450	457	
FT	STRAND	462	466	
FT	STRAND	469	471	
FT	STRAND	490	493	

FT	TURN	496	498
FT	STRAND	501	506
FT	STRAND	509	514
FT	HELIX	517	520
FT	HELIX	526	531
FT	HELIX	534	536
FT	STRAND	538	540
FT	STRAND	545	548
FT	STRAND	552	554
FT	STRAND	557	559
FT	STRAND	564	572
FT	STRAND	580	586
FT	STRAND	590	592
FT	STRAND	595	598
FT	STRAND	602	605
FT	HELIX	614	616
FT	STRAND	619	625
FT	STRAND	637	641
FT	STRAND	646	650
FT	TURN	1048	1052
FT	HELIX	1055	1057
FT	HELIX	1060	1066
FT	HELIX	1067	1069
FT	HELIX	1073	1075
FT	STRAND	1076	1087
FT	STRAND	1090	1097
FT	STRAND	1100	1102
FT	STRAND	1105	1112
FT	HELIX	1118	1132
FT	STRAND	1144	1146
FT	STRAND	1149	1151
FT	STRAND	1154	1158
FT	HELIX	1165	1170
FT	TURN	1172	1174
FT	HELIX	1178	1197
FT	HELIX	1207	1209
FT	STRAND	1210	1212
FT	STRAND	1218	1220
FT	HELIX	1224	1226
FT	HELIX	1232	1234
FT	HELIX	1237	1239
FT	STRAND	1241	1245
FT	HELIX	1247	1249
FT	HELIX	1252	1257
FT	HELIX	1262	1277
FT	STRAND	1285	1287
FT	TURN	1289	1291
FT	HELIX	1292	1297
FT	HELIX	1310	1319
FT	HELIX	1324	1326
FT	HELIX	1330	1342
FT	TURN	1354	1358

SEQ ID No. 48: Anti-MTH1 siRNA

CGACGACAGCUACUGGUUU

SEQ ID No. 49: qPCR-oligonucleotide for detecting p21

5'-CTGTGATGCGCTAATGGCG-3'

SEQ ID No. 50: pPCR-oligonucleotide for detecting p21

5'-AAGTCGAAGTTCCATCGCTCA-3'

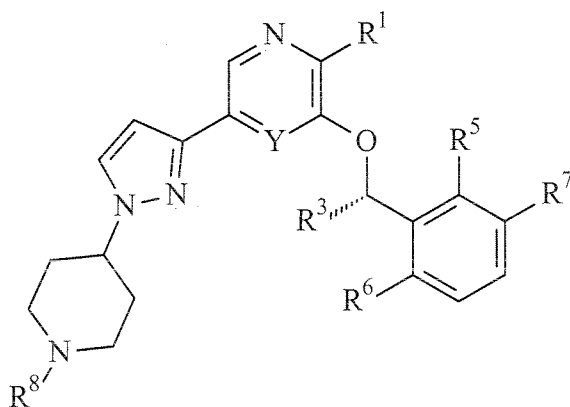
References:

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Claims

1. An (*S*)-enantiomer of an aminoheteroaryl compound for use in treating and/or preventing cancer in a subject, wherein the compound has the following chemical structure represented by Formula (1)



Formula (1)

wherein:

R^1 is $-NH_2$, $-NR^2H$, $-OH$ or $-SH$;

R^2 is C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl;

R^3 is C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl or cyclopropyl;

Y is N or CR^4 ;

R^4 is hydrogen, halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl or C_{3-6} cycloalkyl;

each R^5 , R^6 and R^7 is independently fluorine, chlorine, bromine or iodine;

R^8 is hydrogen or $-A-B_n-X$, wherein

A is a single bond, $-C(=O)-$ or $-C(=O)CH_2-$;

B is C_{1-4} alkylene, C_{2-4} alkenylene or $-(OCH_2CH_2)-$;

n is 0, 1, 2, 3, 4 or 5, and

X is $-NHR^2$; $-NH_2$; $-SH$; $-OH$ or O-alkyl;

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

2. The compound for the use according to claim 1, wherein

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R^1 is $-NH_2$, $-OH$ or SH ;

R^3 is methyl;

Y is CR^4 ;

R^4 is hydrogen or halogen;

each R^5 , R^6 and R^7 is independently fluorine or chlorine;

R^8 is hydrogen or $-A-B_n-X$, wherein

B is C_{1-4} alkylene or $-(OCH_2CH_2)-$;

n is 1, 2 or 3, and

X is $-NH_2$;

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

3. The compound for the use according to claim 1 or 2, wherein

R^8 is hydrogen;

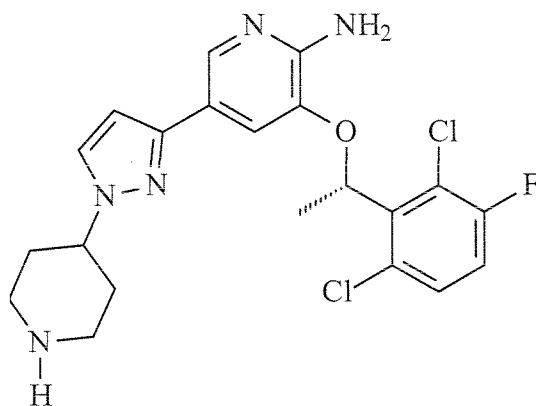
$-C(=O)CH_2CH_2CH_2CH_2CH_2-NH_2$;

$-C(=O)CH_2-O-CH_2CH_2-O-CH_2CH_2-NH_2$; or

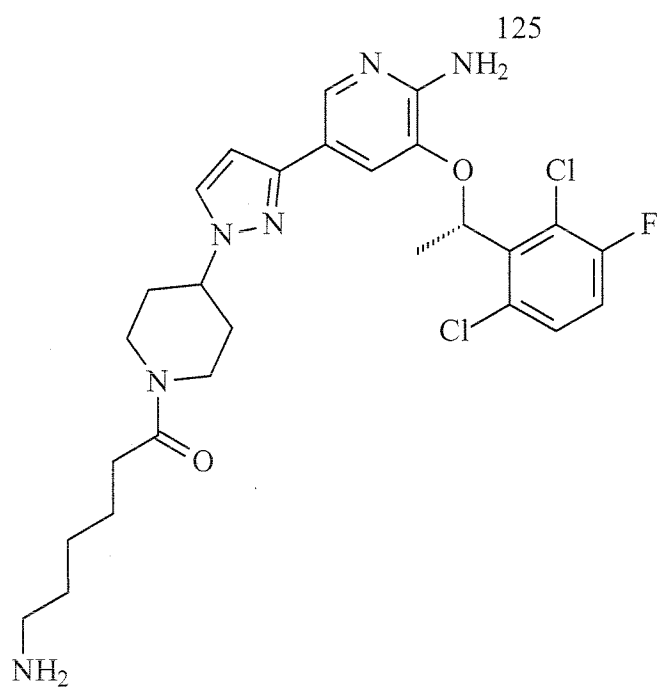
$-CH_2CH_2CH_2-NH_2$;

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

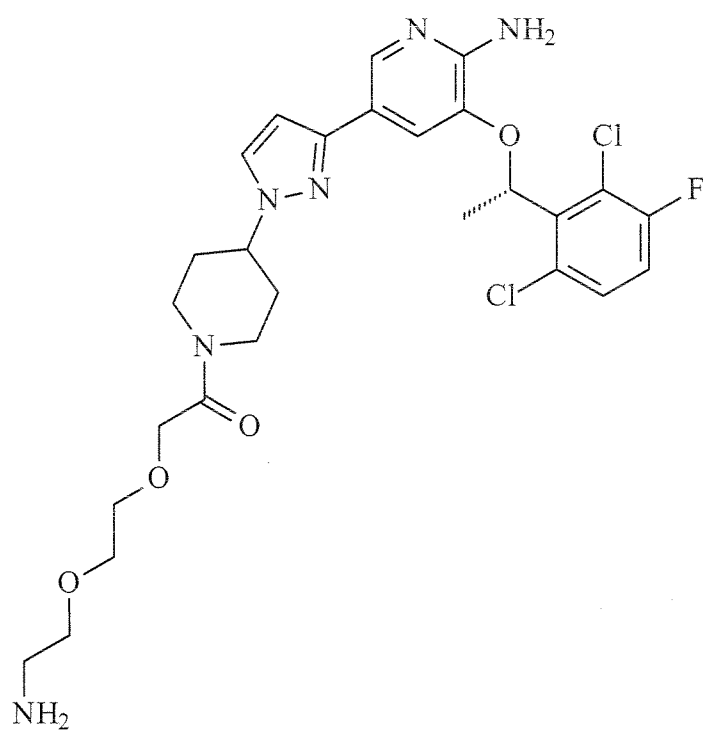
4. The compound for the use according to claim 1, wherein the compound has one of the following chemical structures represented by Formulae (2) to (5):



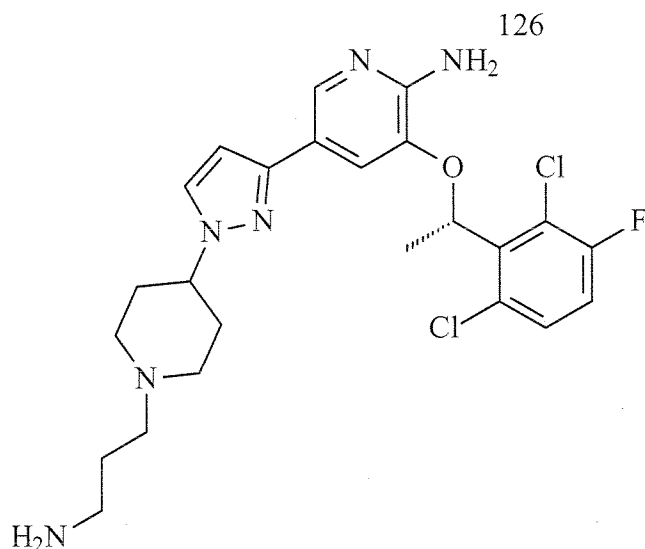
Formula (2);



Formula (3);



Formula (4) or



Formula (5).

5. A pharmaceutical composition for use in treating and/or preventing cancer, wherein said pharmaceutical composition comprises the compound according to any one of claims 1 to 4 and a pharmaceutically acceptable excipient.
6. The pharmaceutical composition of claim 5, comprising at least two components each having one of the chemical structures represented by Formulae (1) to (5).
7. The pharmaceutical composition of claim 6, comprising at least two components each having one of the chemical structures represented by Formulae (2) to (5).
8. The compound for the use according to any one of claims 1 to 4, or the pharmaceutical composition of any one of claims 5 to 7, wherein the cancer cell or tissue of said subject has an activating RAS mutation and/or an activating EGFR mutation.
9. The compound for the use according to any one of claims 1 to 4 and 8, or the pharmaceutical composition of any one of claims 5 to 8, wherein the cancer cell or tissue of said subject has an activating RAS mutation.
10. The compound for the use according to claims 8 or 9, or the pharmaceutical composition of claim 8 or 9, wherein said activating RAS mutation is an activating KRAS mutation.

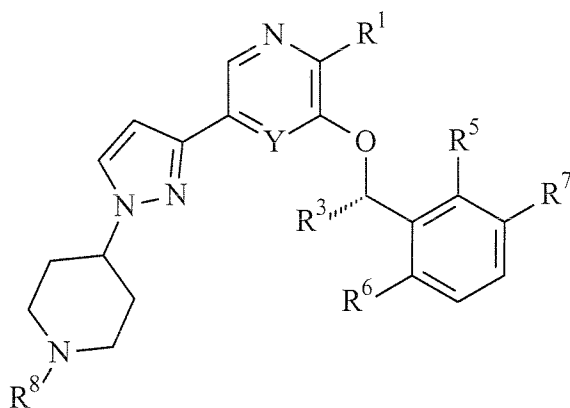
11. The compound for the use according to any one of claims 1 to 4 and 8 to 10, or the pharmaceutical composition of any one of claims 5 to 10, wherein the treatment and/or prevention of cancer in a subject is independent of the ALK status and/or the c-Met status of the cancer cell or tissue of said subject.
12. The compound for the use according to claim 11, or the pharmaceutical composition of claim 11, wherein said ALK-status is the level of ALK biological activity and/or the level of ALK expression.
13. The compound for the use according to claim 11, or the pharmaceutical composition of claim 11, wherein said c-Met-status is the level of c-Met biological activity and/or the level of c-Met expression.
14. The compound for the use according to any one of claims 1 to 4 and 8 to 13, or the pharmaceutical composition of any one of claims 5 to 13, wherein the cancer cell or tissue of said subject does not have a gene mutation and/or a chromosomal translocation of ALK.
15. The compound for the use according to claim 14, or the pharmaceutical composition of claim 14, wherein said chromosomal translocation is EML4-ALK.
16. The compound for the use according to any one of claims 1 to 4 and 8 to 15, or the pharmaceutical composition of any one of claims 5 to 15, wherein in the cancer cell or tissue of said subject the biological activity and/or expression of p21 is reduced or absent.
17. The compound for the use according to any one of claims 1 to 4 and 8 to 16, or the pharmaceutical composition of any one of claims 5 to 16, wherein said subject is a mammal.
18. The compound for the use according to claim 17, or the pharmaceutical composition of claim 17, wherein said mammal is a human patient.

19. The compound for the use according to any one of claims 1 to 4 and 8 to 18, or the pharmaceutical composition of any one of claims 5 to 18, wherein said cancer is selected from the group consisting of colon cancer, lung cancer, breast cancer, leukaemia, lymphoma, skin cancer, liver cancer, pancreatic cancer, brain cancer, kidney cancer, neuroblastoma, Ewing's sarcoma, prostate cancer, bladder cancer and esophagus cancer.
20. The compound for the use according to claim 19, or the pharmaceutical composition of claim 19, wherein said breast cancer lacks expression of estrogen receptor- α and progesterone receptor and lacks overexpression or amplification of the HER2/NEU oncogene.
21. The compound for the use according to claim 19 or 20, or the pharmaceutical composition of claim 19 or 20, wherein said breast cancer has a BRCA1 and/or BRCA2 gene mutation.
22. The compound for the use according to any one of claims 1 to 4 and 8 to 21, or the pharmaceutical composition of any one of claims 5 to 21, wherein said compound or pharmaceutical composition is co-administered with radiation and/or chemotherapy.
23. The compound for the use according to claim 22, or the pharmaceutical composition of claim 22, wherein said chemotherapy is a DNA damaging drug, an alkylating agent, a DNA intercalator, a topoisomerase inhibitor, an agent which confers oxidative damage to DNA, a cytotoxic compound, an antimetabolite, a compound which interferes with DNA repair mechanisms, an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 kinase inhibitor, a PARP inhibitor, an EGFR inhibitor, a DNA-dependent protein kinase inhibitor, a generic base excision repair inhibitor, a DNA polymerase beta inhibitor, a O-6-methylguanine methyltransferase (MGMT) inhibitor, a survivin suppressant, a compound generating reactive oxygen species (ROS), an antimitotic compound, or a combination of any of the foregoing.
24. The compound for the use according to claim 23, or the pharmaceutical composition of claim 23, wherein said chemotherapy is a PARP inhibitor and/or an EGFR inhibitor.

25. The compound for the use according to claim 22 or 23, or the pharmaceutical composition of claim 22 or 23, wherein said chemotherapy is cyclophosphamide, temozolomide, melphalan, carmustine, busulfan, cisplatin, procarbazine, anthracyclines, camptothecin, irinotecan, etoposide, hydrogen peroxide, resorcinol, quinones, methotrexate, 5-fluorouracil, thalidomide, lenalidomide, pomalidomide, olaparib, ABT-888, neocarzinostatin, bleomycin, decitabine, 5-azacytosine, methoxyamine hydrochloride (TRC102), lomeguatrib, piperlongumine, quercetin, vincristin, taxol, mitoxantrone, YM155, erlotinib, gefitinib, lapatinib, or a combination of any of the foregoing.
26. *In vitro* method for determining the effectiveness of the compound for the use according to any one of claims 1 to 4 and 8 to 25, or the pharmaceutical composition of any one of claims 5 to 25, the method comprising the steps of:
- (a) obtaining a cell or tissue sample from a subject; and
 - (b) determining the subject's NUDT1/MTH1-status;
- wherein a NUDT1/MTH1-positive cell or tissue sample is indicative of an effective treatment and/or prevention of cancer.
27. The *in vitro* method of claim 26, wherein said NUDT/MTH1-status is the level of MTH1 biological activity and/or the level of MTH1 expression.
28. The *in vitro* method of claim 26 or 27, further comprising the step of:
- (c) determining the subject's RAS-status;
- wherein a cell or tissue sample positive for NUDT1/MTH1 and positive for an activating RAS mutation are indicative of an effective treatment and/or prevention of cancer.
29. The *in vitro* method of claim 26 or 27, further comprising the step of:
- (c) determining whether said cell or tissue sample has an activating RAS mutation;
- wherein a cell or tissue sample positive for NUDT1/MTH1 and positive for an activating RAS mutation are indicative of an effective treatment and/or prevention of cancer.

30. The *in vitro* method of any one of claims 26 to 29, further comprising the step of:
(d) determining whether in said cell or tissue sample the expression and/or biological activity of p21 is reduced or absent;
wherein a cell or tissue sample which is positive for NUDT1/MTH1 and wherein the expression and/or biological activity of p21 is reduced or absent are indicative of an effective treatment and/or prevention of cancer.
31. The *in vitro* method of any one of claims 26 to 30, wherein said cell or tissue sample is a cancer cell or tissue.
32. The *in vitro* method of any one of claims 26 to 31, wherein step (b) and/or (c) and/or (d) comprises at least one detection method selected from the group consisting of PCR (polymerase chain reaction), gene sequencing, ARMS (Amplification Refractory Mutation System), Peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, PCR-Invader, SNaPshot, PCR/HRMA/dHPLC, PCR/fIRFLP, Fluorescent In-Situ Hybridisation (FISH), Immunohistochemistry (IHC), RT-PCR, gene arrays, and gene chips.
33. Use of a kit in the *in vitro* method as defined in any one of claims 26 to 32, the kit comprising polynucleotides and/or antibodies capable of detecting NUDT1/MTH1.
34. The use of claim 33, wherein the kit further comprises polynucleotides and/or antibodies capable of detecting RAS.
35. The use of claim 33 or 34, wherein the kit further comprises polynucleotides and/or antibodies capable of detecting p21.
36. A screening method for identifying a target of an (*S*)-enantiomer of an aminoheteroaryl compound having the following chemical structure represented by Formula (1),

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Formula (1)

wherein:

R^1 is $-NH_2$, $-NR^2H$, $-OH$ or $-SH$;

R^2 is C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl;

R^3 is C_{1-3} alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl or cyclopropyl;

Y is N or CR^4 ;

R^4 is hydrogen, halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl or C_{3-6} cycloalkyl;

each R^5 , R^6 and R^7 is independently fluorine, chlorine, bromine or iodine;

R^8 is hydrogen or $-A-B_n-X$, wherein

A is a single bond, $-C(=O)-$ or $-C(=O)CH_2-$;

B is C_{1-4} alkylene, C_{2-4} alkenylene or $-(OCH_2CH_2)-$;

n is 0, 1, 2, 3, 4 or 5, and

X is $-NHR^2$; $-NH_2$; $-SH$; $-OH$ or O -alkyl;

or a pharmaceutically acceptable salt, solvate or prodrug thereof;

and wherein the screening method comprises the steps of:

- (a) obtaining a cell lysate;
 - (b) contacting said aminoheteroaryl compound with said cell lysate; and
 - (c) determining whether a molecule binds to said aminoheteroaryl compound,
- wherein, if a molecule is found to bind to said aminoheteroaryl compound, then such molecule is identified as a target of said aminoheteroaryl compound.

37. The screening method of claim 36, wherein in compound of Formula (1)

R^1 is $-NH_2$, $-OH$ or $-SH$;

R^3 is methyl;

Y is CR^4 ;

R^4 is hydrogen or halogen;

each R^5 , R^6 and R^7 is independently fluorine or chlorine;

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R^8 is hydrogen or $-A-B_n-X$, wherein

B is C_{1-4} alkylene or $-(OCH_2CH_2)-$;

n is 1, 2 or 3, and

X is $-NH_2$.

38. The screening method of claim 36 or 37, wherein in compound of Formula (1)

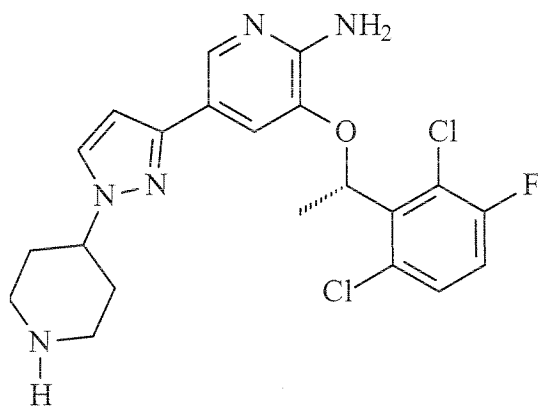
R^8 is hydrogen;

$-C(=O)CH_2CH_2CH_2CH_2CH_2-NH_2$;

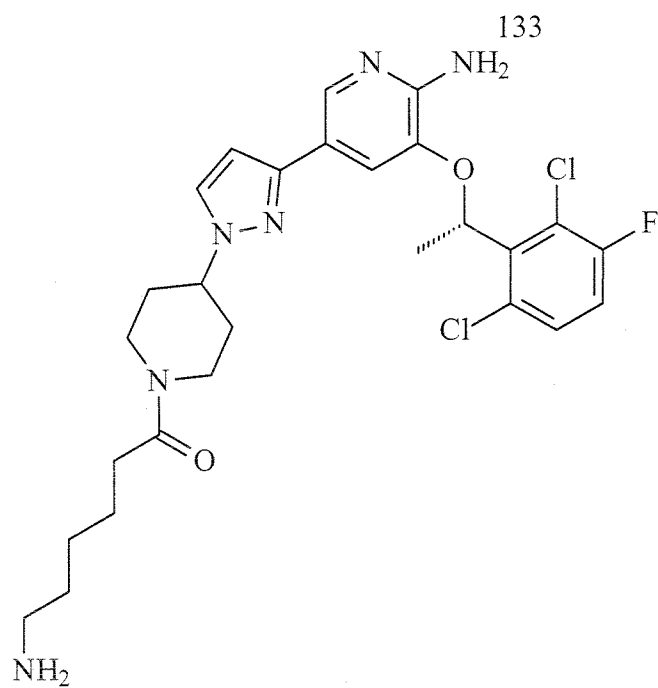
$-C(=O)CH_2-O-CH_2CH_2-O-CH_2CH_2-NH_2$; or

$-CH_2CH_2CH_2-NH_2$.

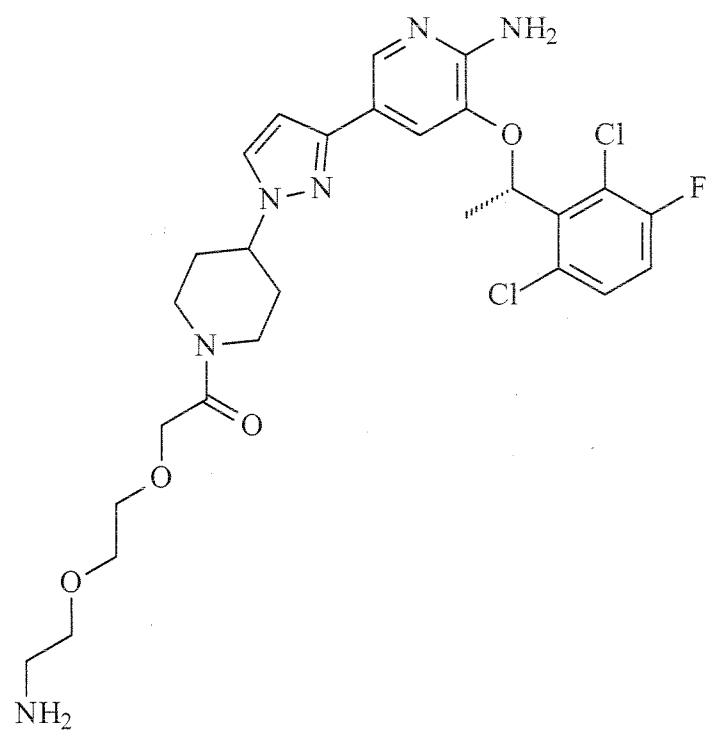
39. The screening method of claim 36, wherein the compound has one of the following chemical structures represented by Formulae (2) to (5):



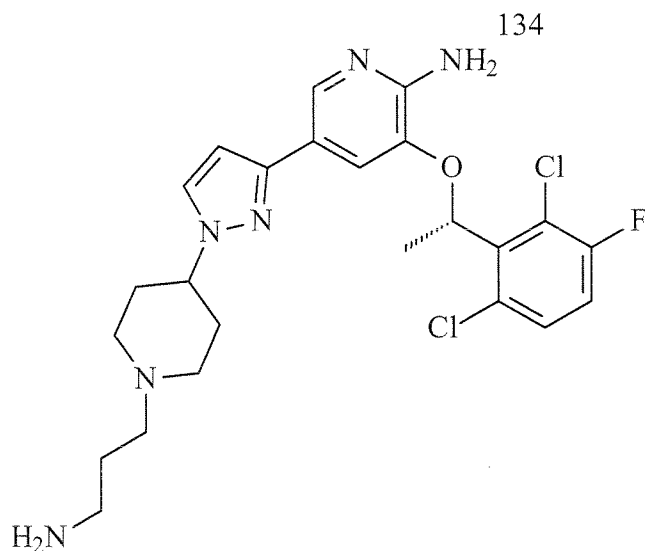
Formula (2);



Formula (3);



Formula (4) or



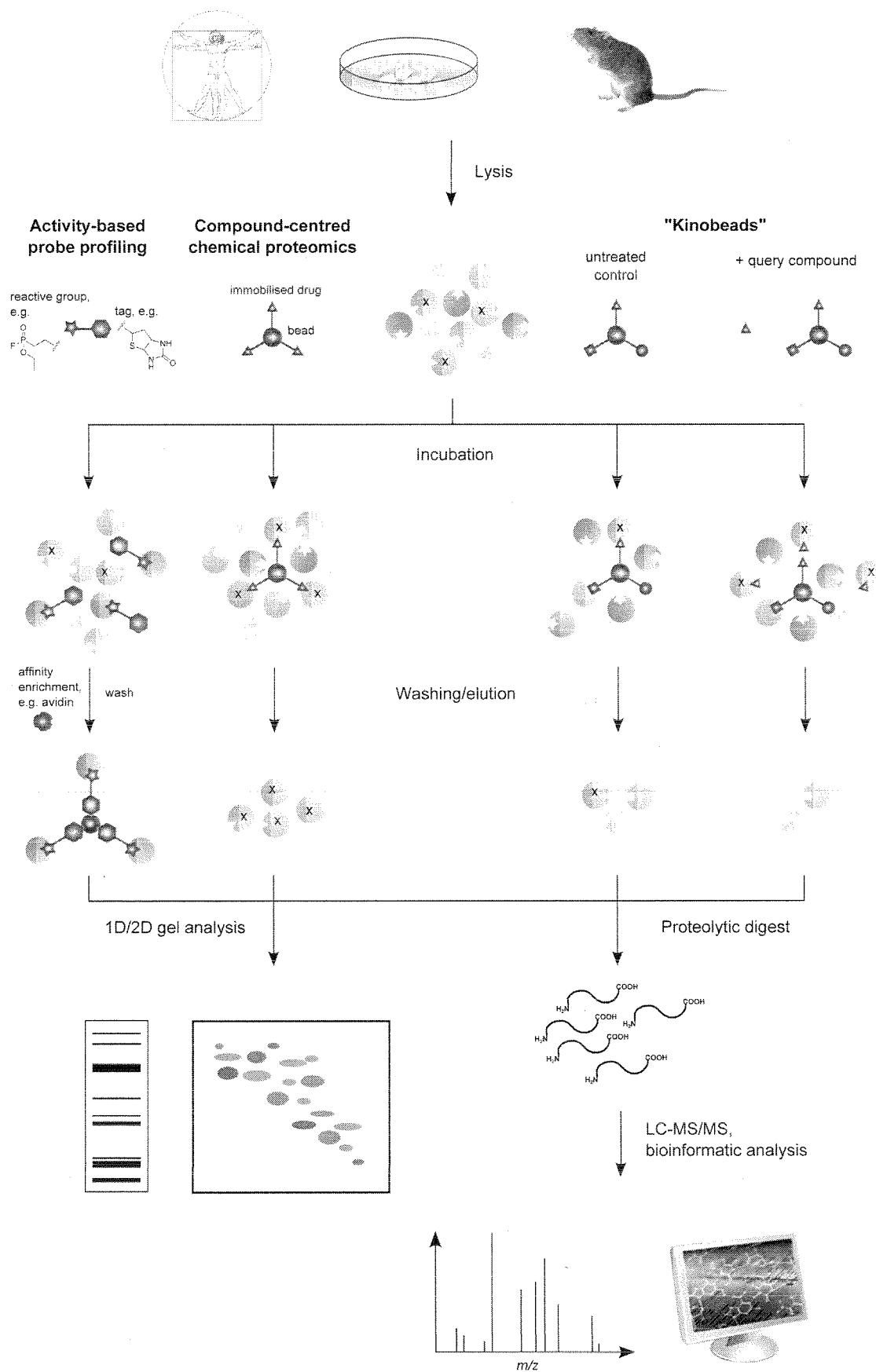
Formula (5).

or a pharmaceutically acceptable salt, solvate or prodrug thereof.

40. The screening method of any one of claims 36 to 39, further comprising the step of:
- (d) determining whether said (*s*)-enantiomer of an aminoheteroaryl compound alters the biological activity of said molecule,
- wherein, if said (*S*)-enantiomer of an aminoheteroaryl compound is found to alter the biological activity of said molecule, then such molecule is identified as a target of said (*S*)-enantiomer of an aminoheteroaryl compound.
41. The screening method of any one of claims 36 to 40, wherein said cell lysate is derived from a cell or tissue sample from a cancer patient, from an established cancer cell line or from a non-human animal.
42. The screening method of claim 40 or 41, wherein altering the biological activity is inhibiting the biological activity.
43. The compound for the use according to any one of claims 1 to 4 and 8 to 25, the pharmaceutical composition of any one of claims 5 to 25, the *in vitro* method of any one of claims 26 to 32, the use of any one of claims 33 to 35, or the screening method of any one of claims 36 to 42, wherein said compound inhibits the biological activity of MTH1.

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Figure 1



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Figure 2

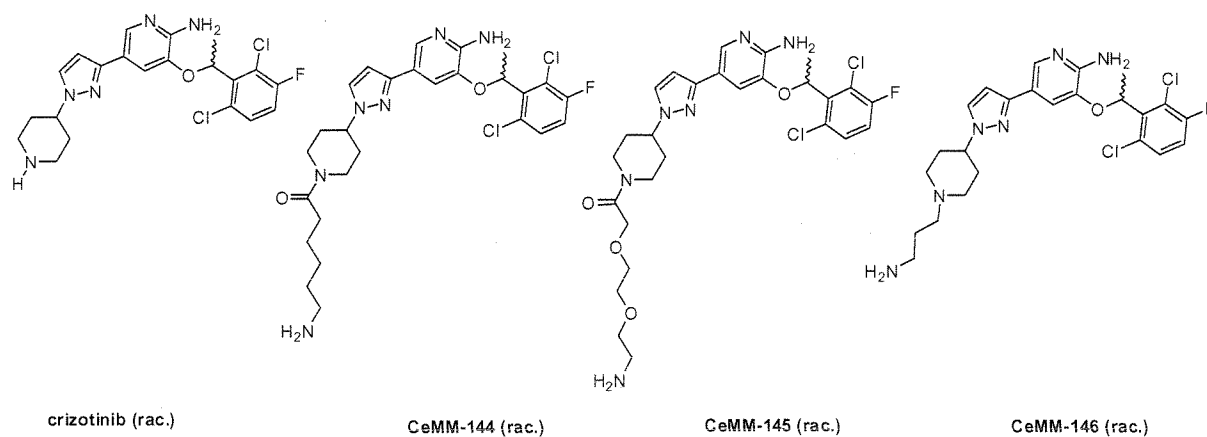


Figure 3

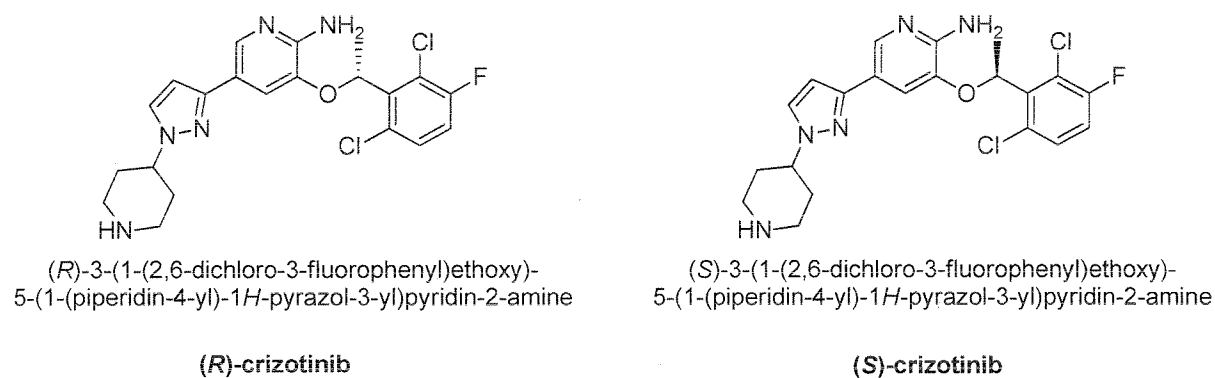
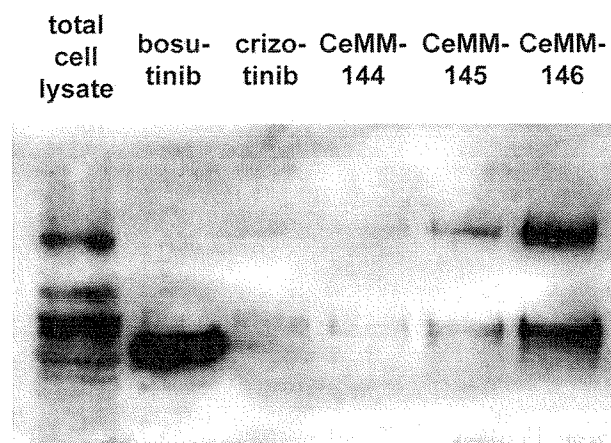
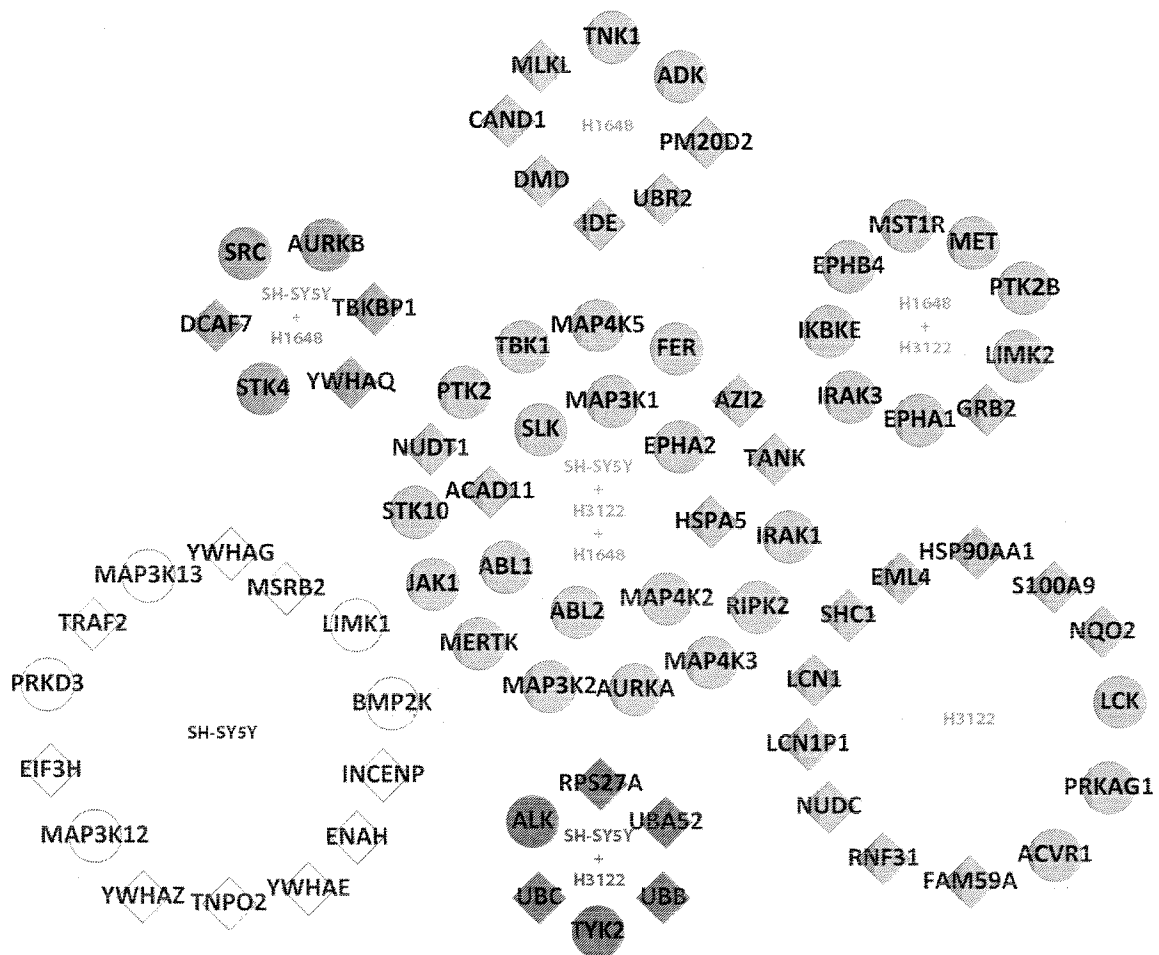


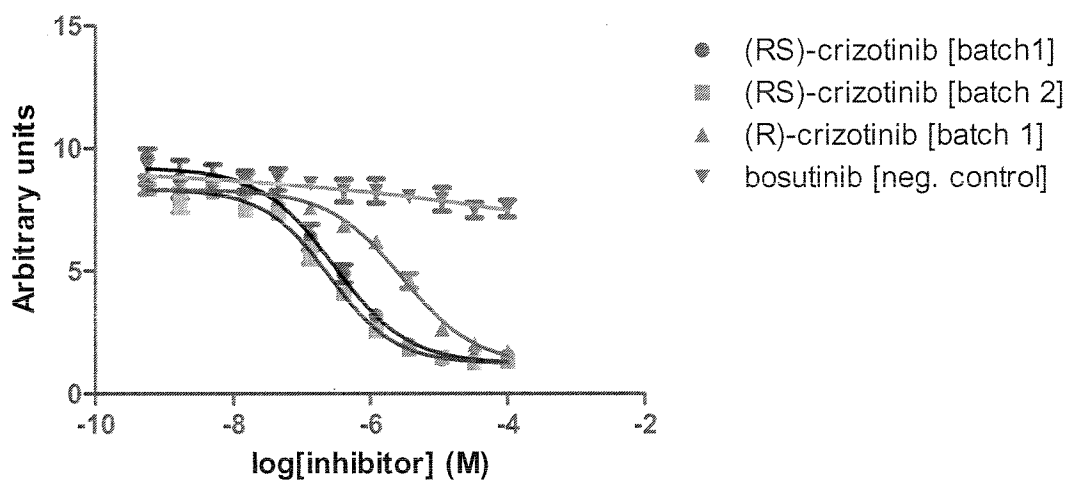
Figure 4





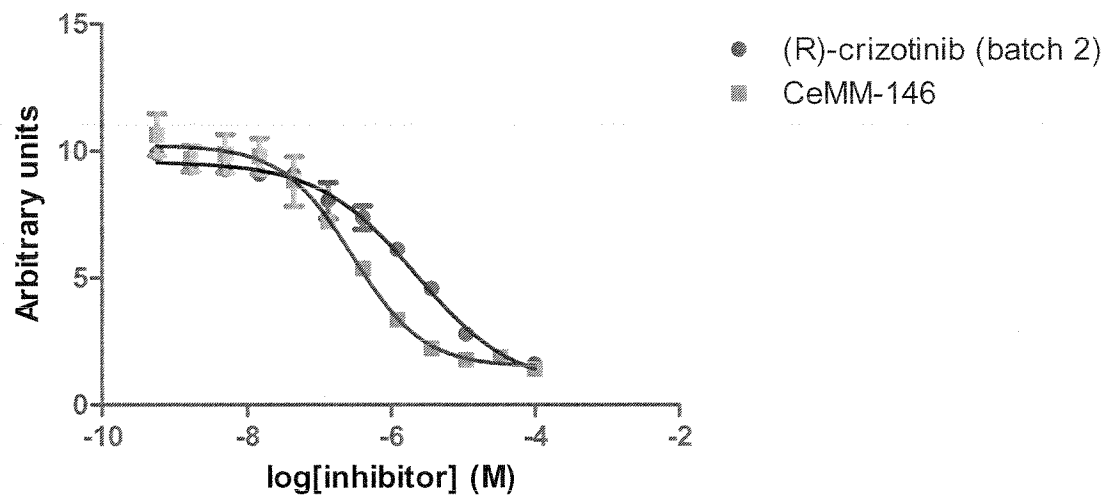
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Figure 6



	(RS)-crizotinib [batch1]	(RS)-crizotinib [batch 2]	(R)-crizotinib [batch 1]	bosutinib [neg. control]
IC50	2.985e-007	2.559e-007	2.906e-006	~ 1.236e+013

Figure 7



	(R)-crizotinib (batch 2)	CeMM-146
IC50	2.247e-006	2.974e-007

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Figure 8

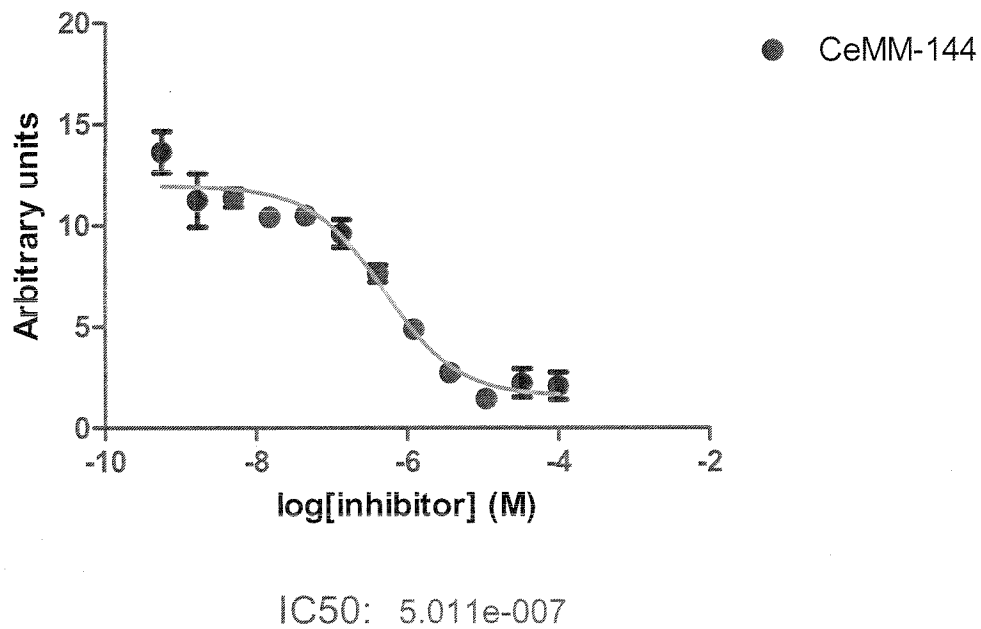
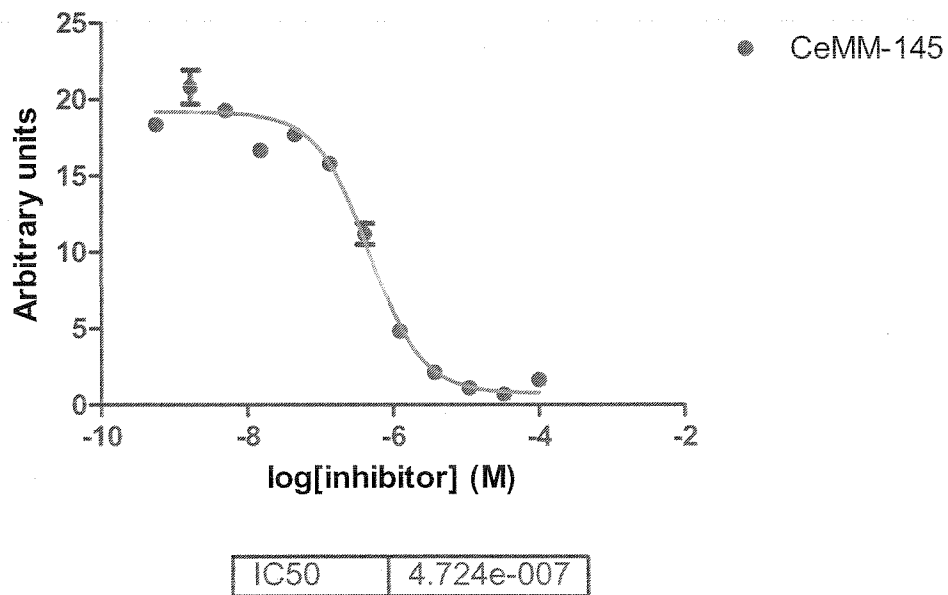


Figure 9



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Figure 10

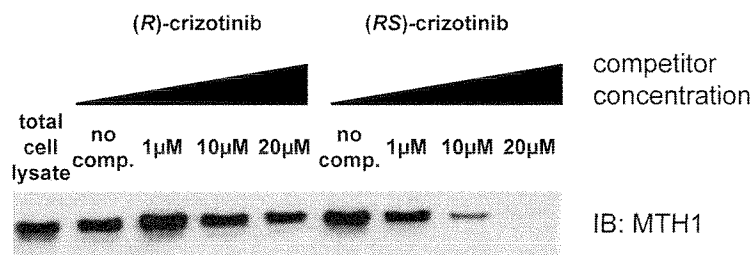
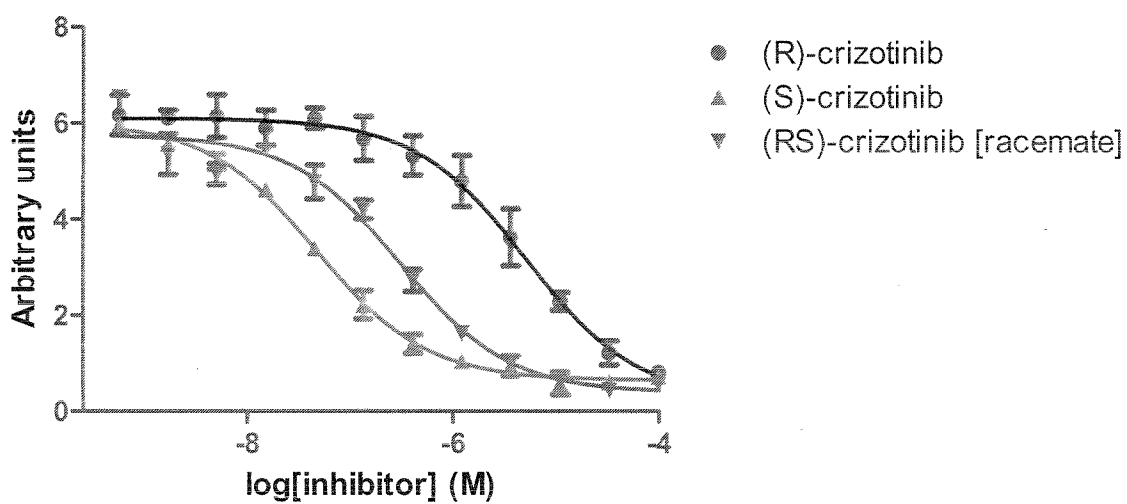


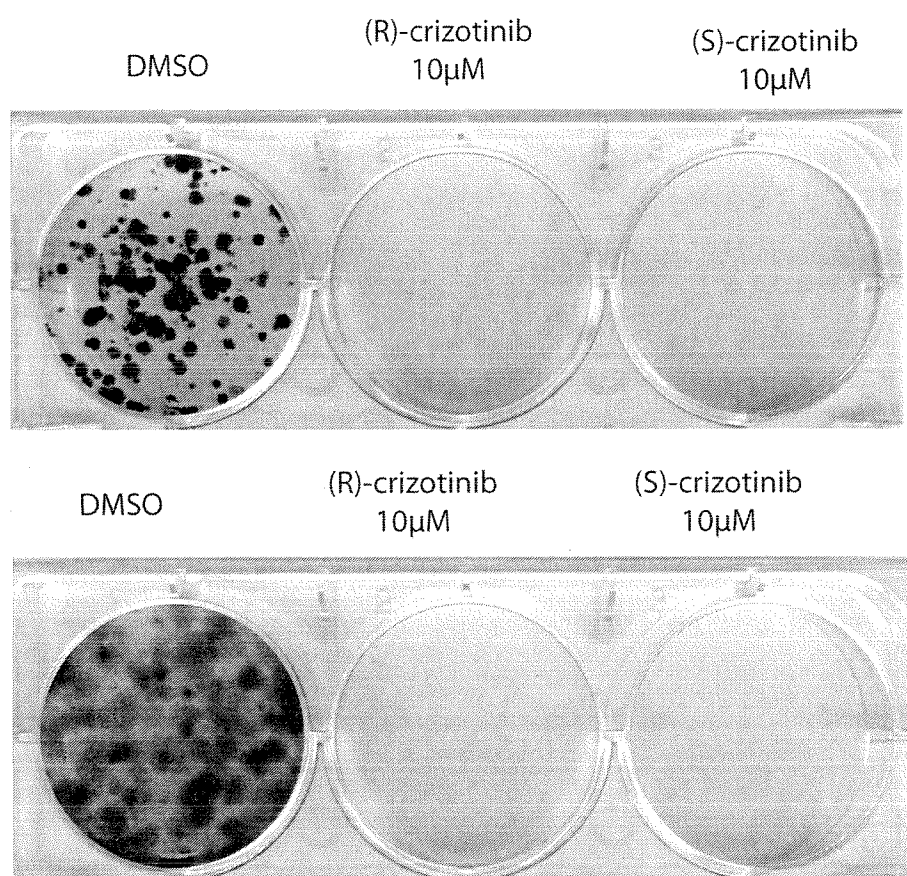
Figure 11



	(R)-crizotinib	(S)-crizotinib	(RS)-crizotinib [racemate]
IC ₅₀	5.236e-006	4.845e-008	3.150e-007

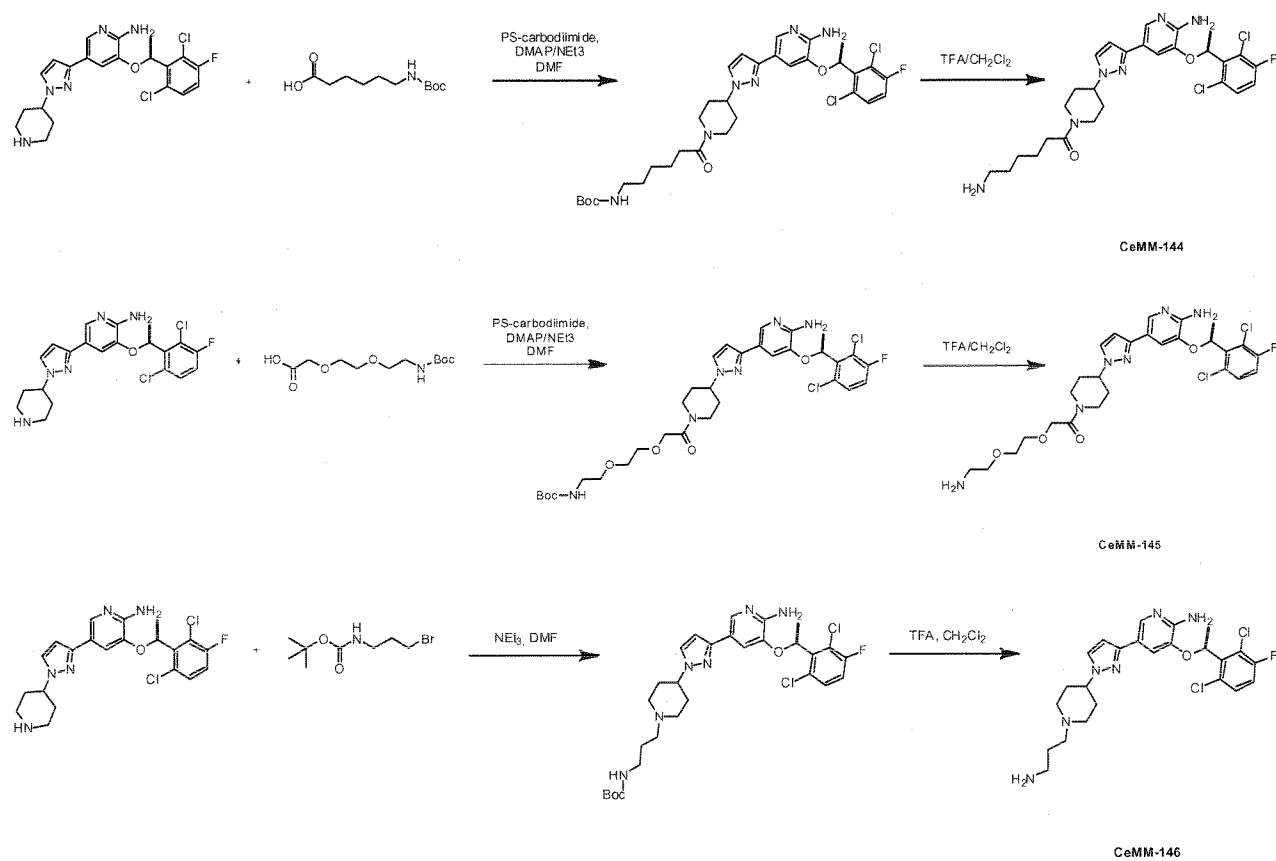
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Figure 12



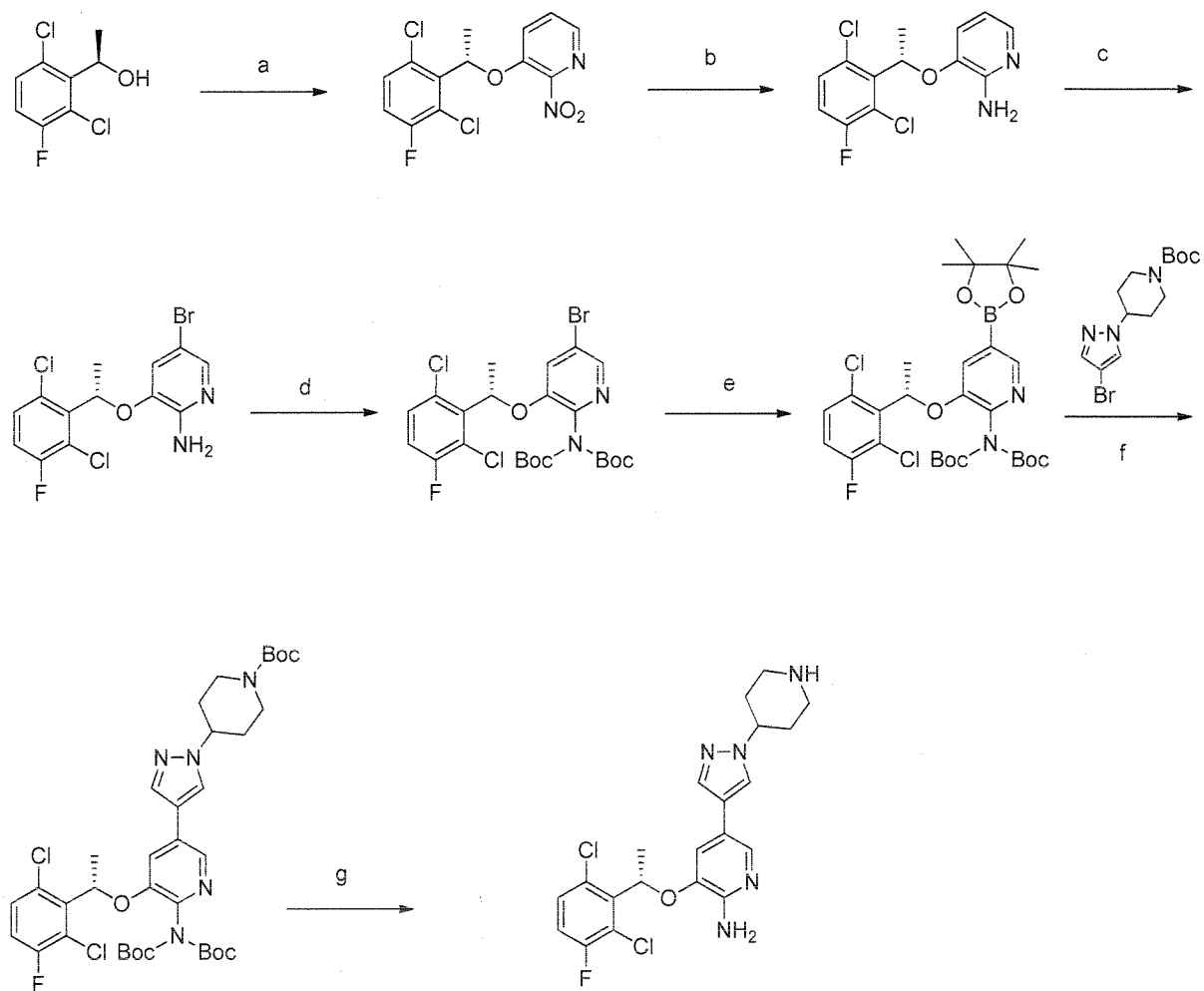
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Figure 13



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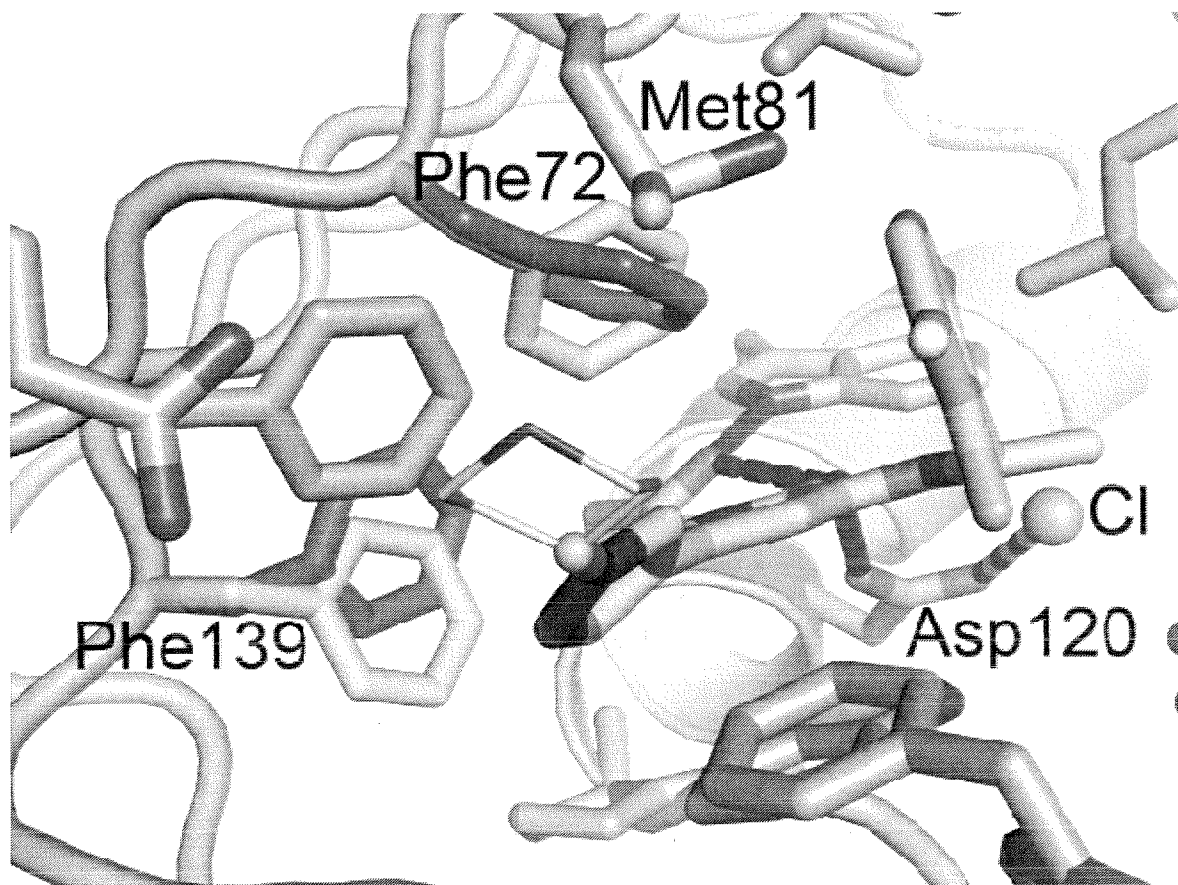
Figure 14



(S)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)-5-(1-(piperidin-4-yl)-1H-pyrazol-4-yl)pyridin-2-amine

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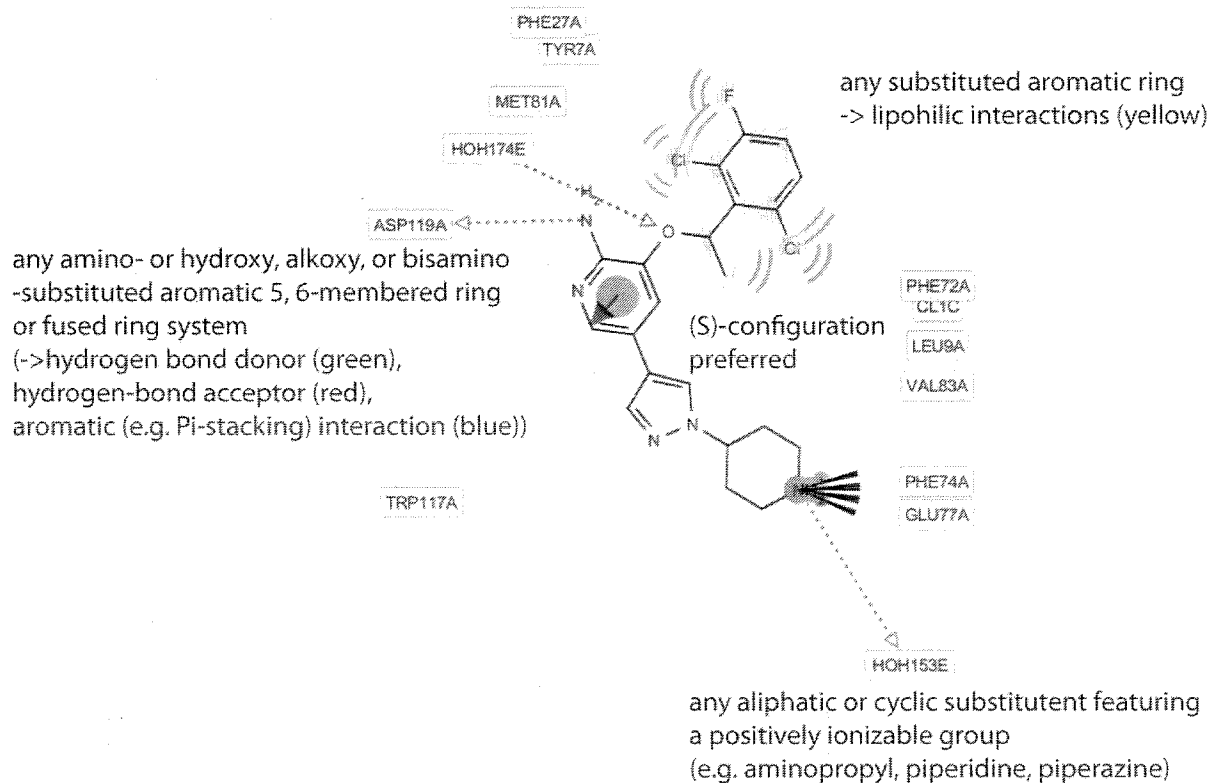
Figure 15



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Figure 16

MTH1 Inhibitor Pharmacophore



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Figure 17

A

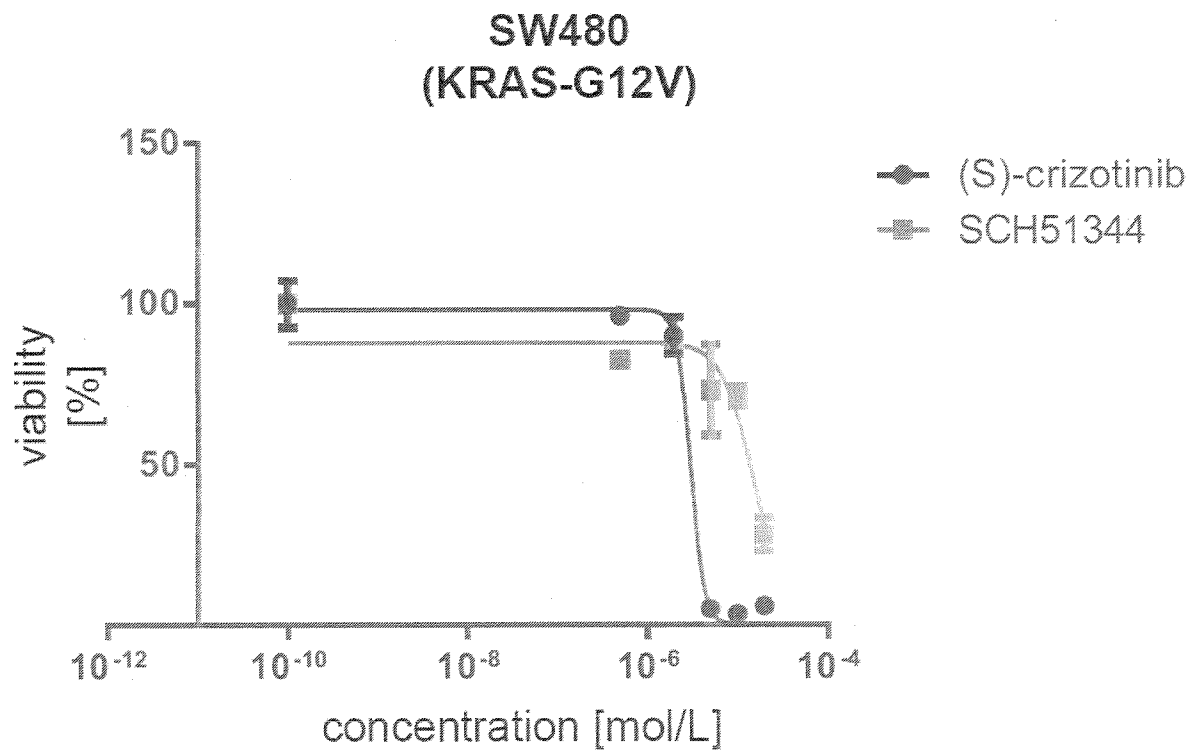
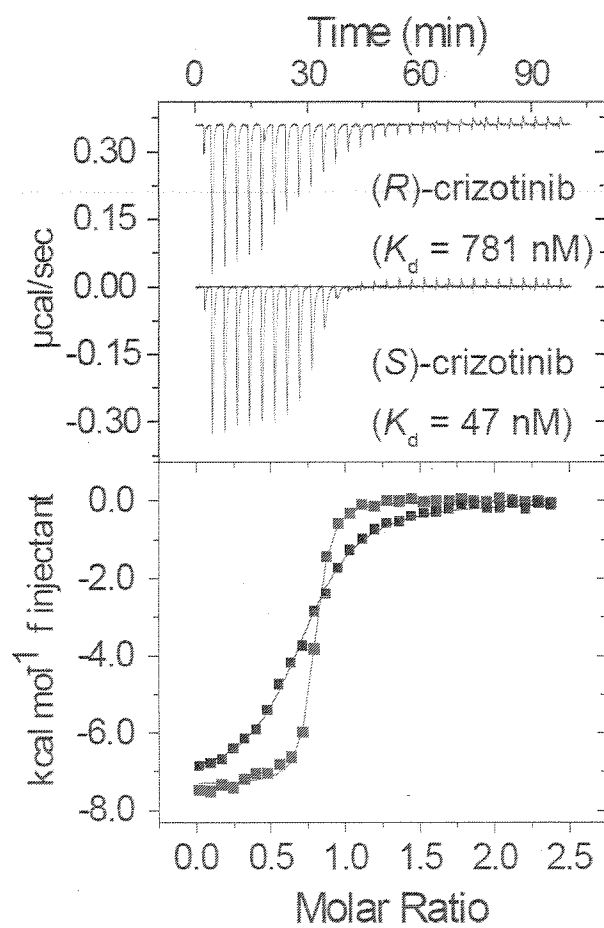
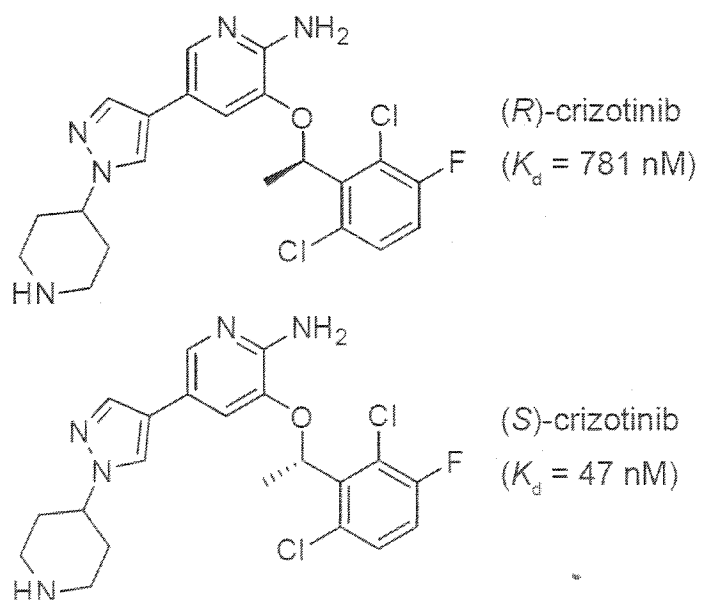


Figure 17 cont.

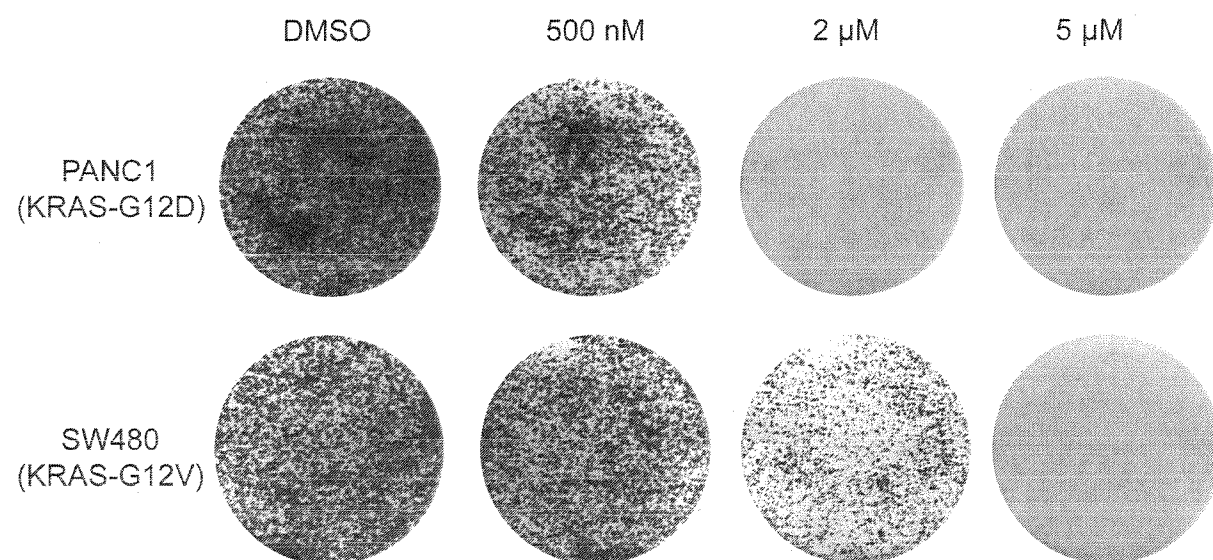
B



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Figure 17 cont.

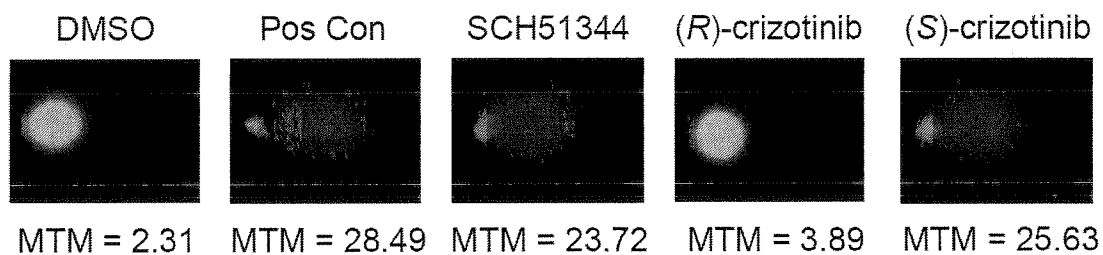
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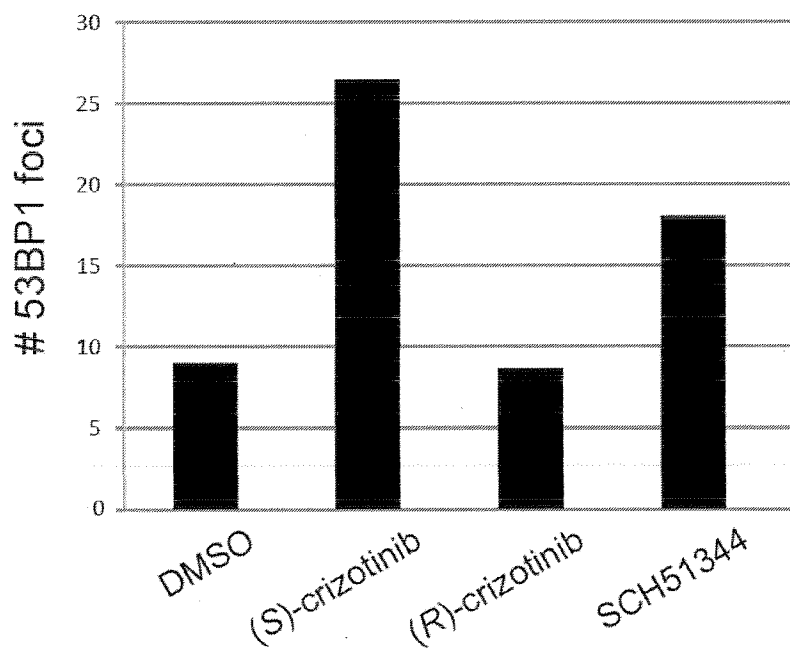
15/27

Figure 18

A



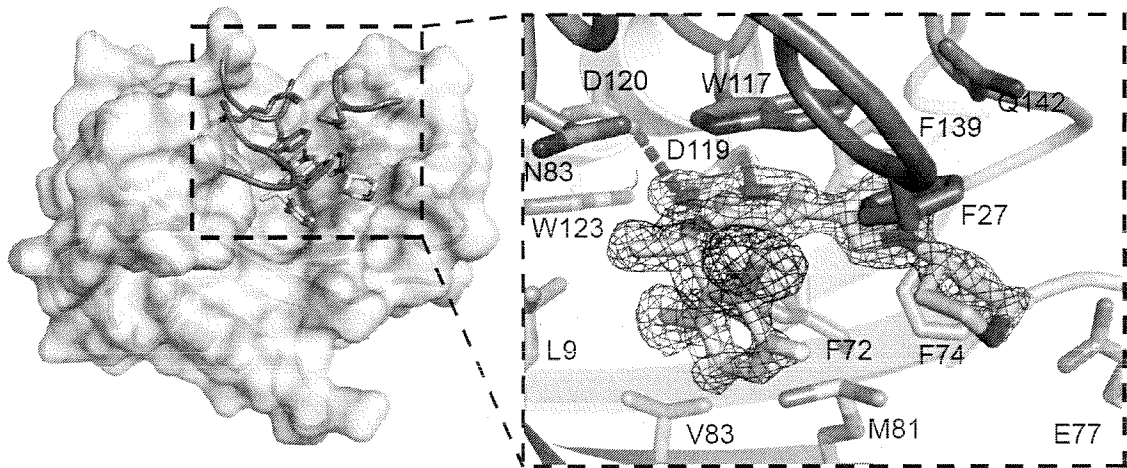
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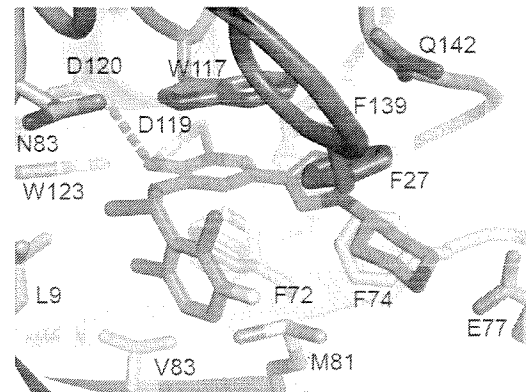
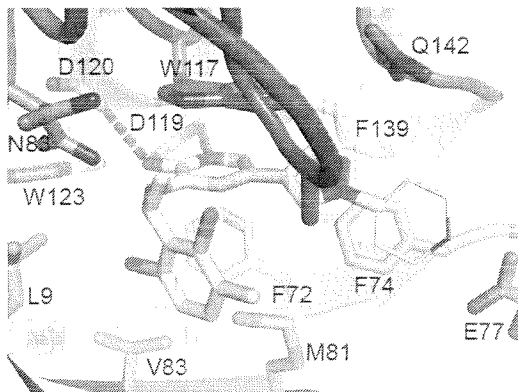
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Figure 18 cont.

C



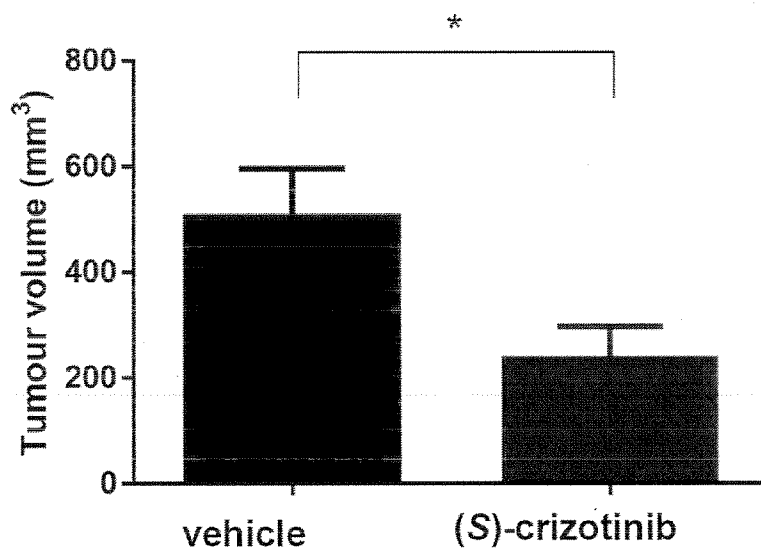
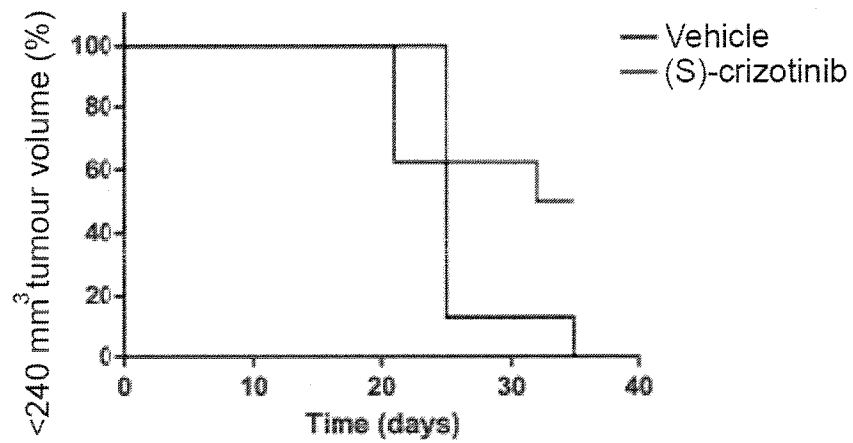
D



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Figure 18 cont.

E



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Figure 19

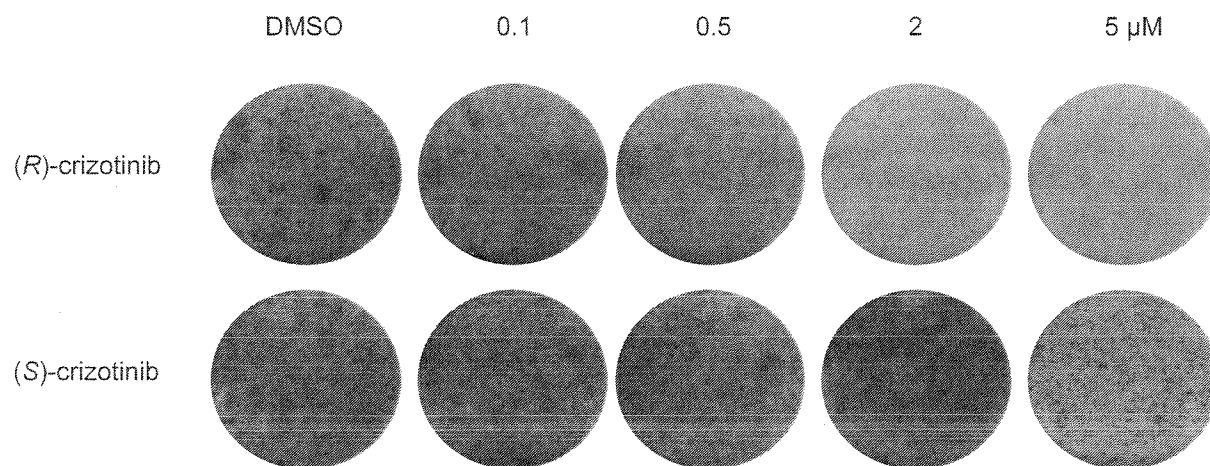
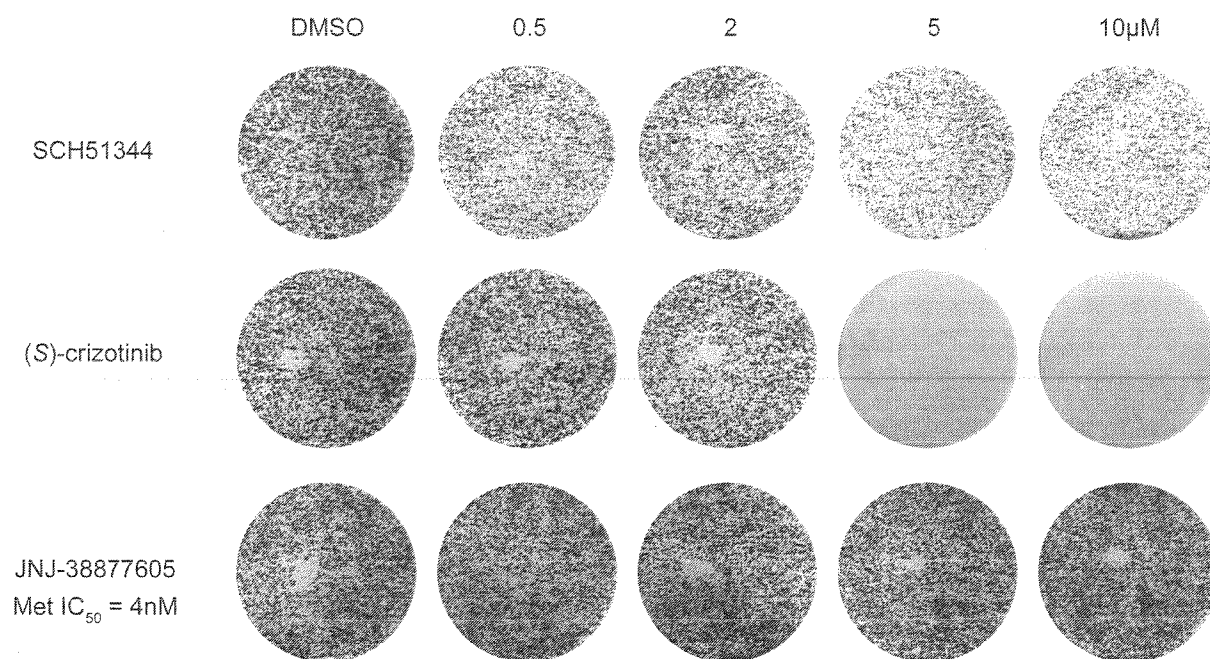
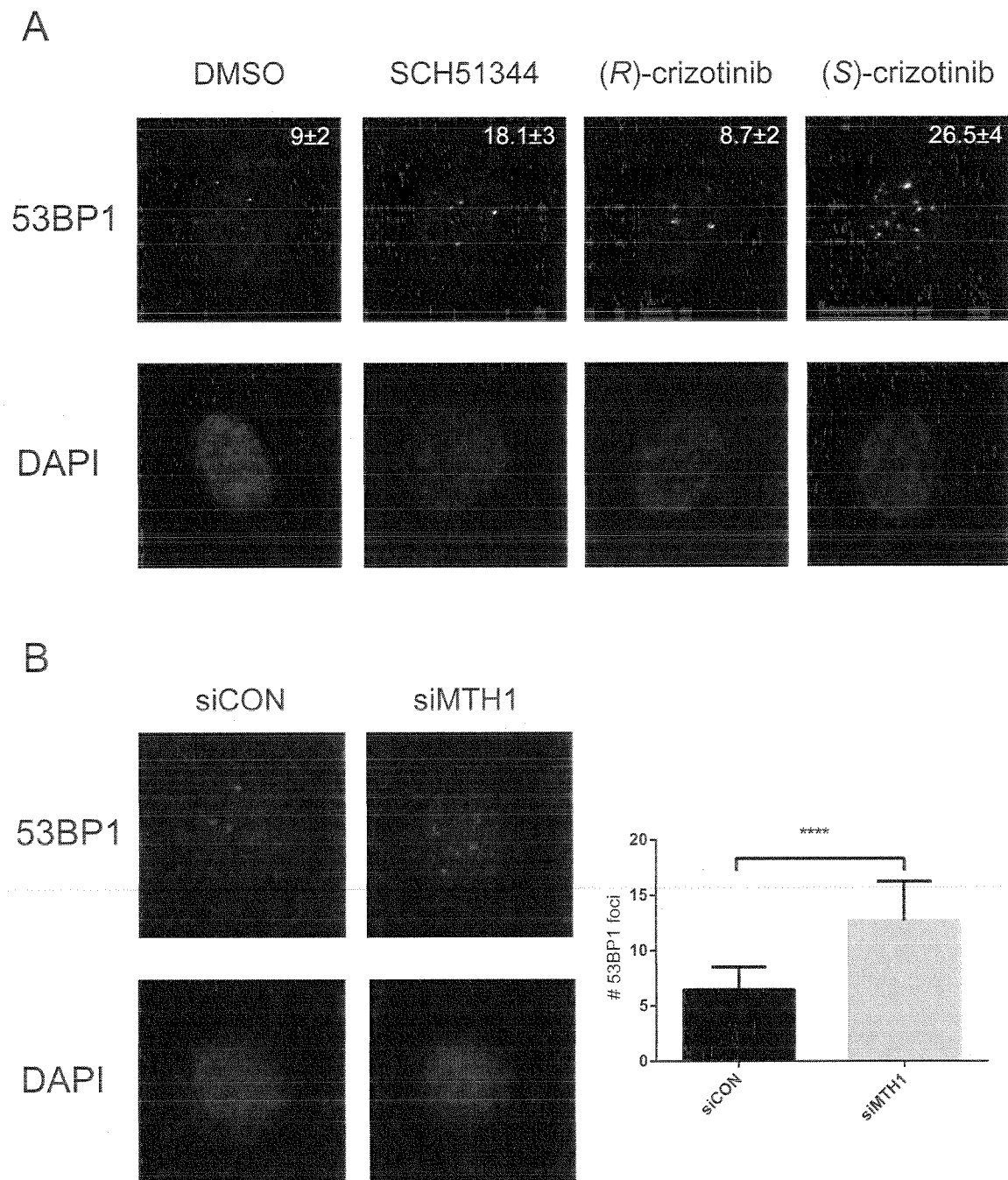


Figure 20



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Figure 21



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Figure 22

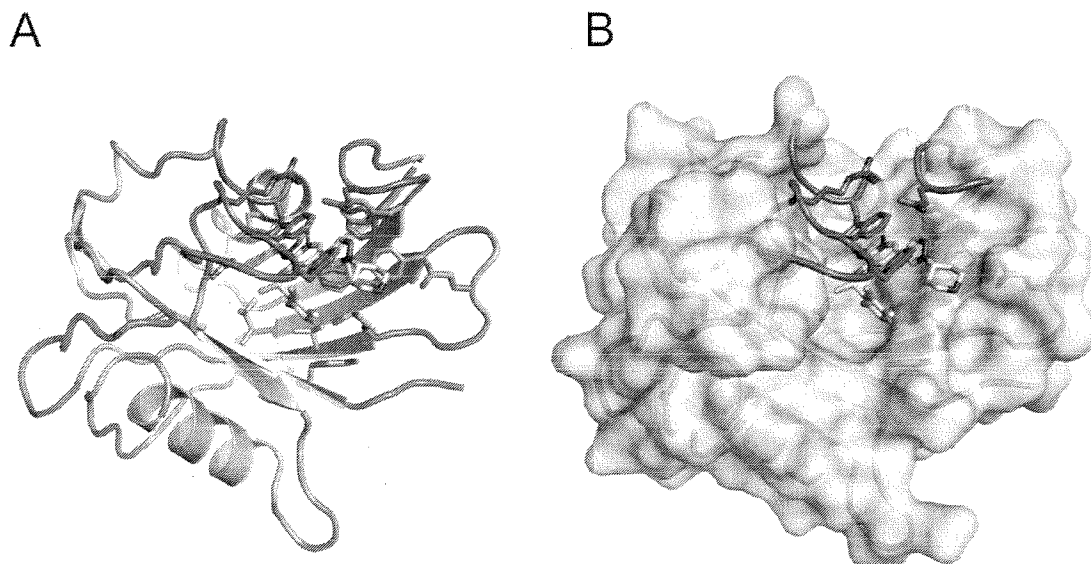


Figure 23

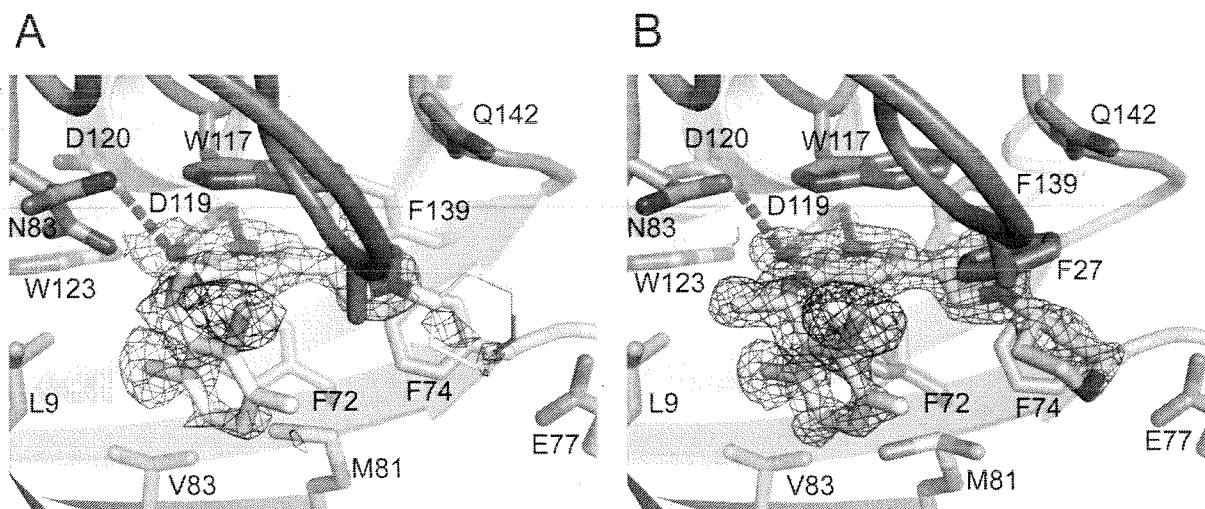


Figure 24

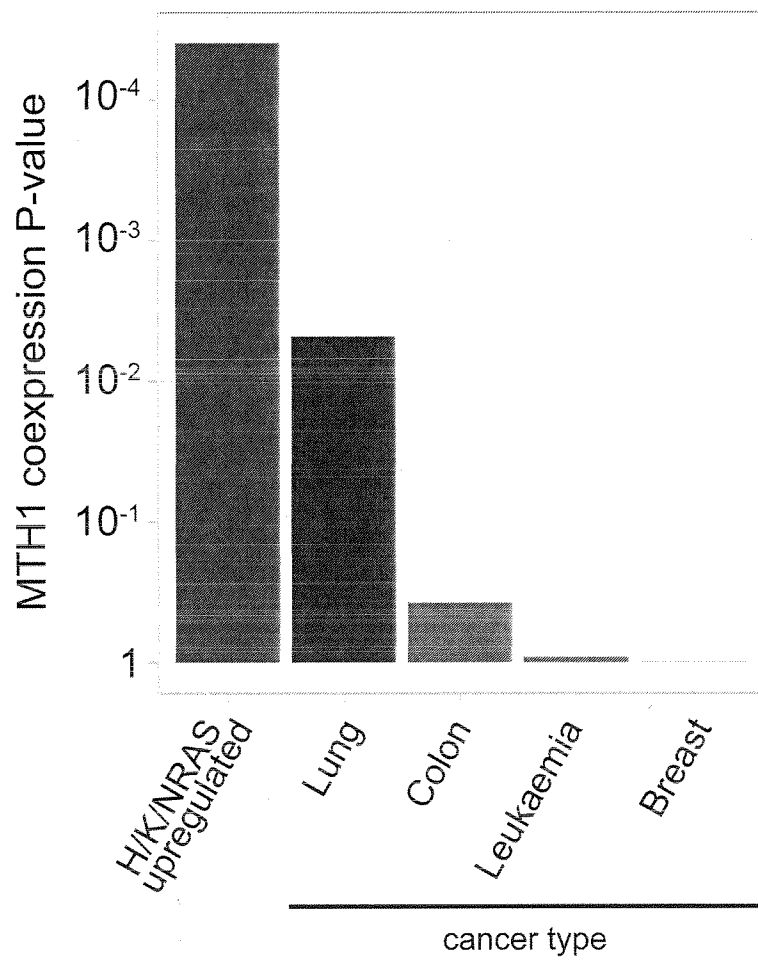
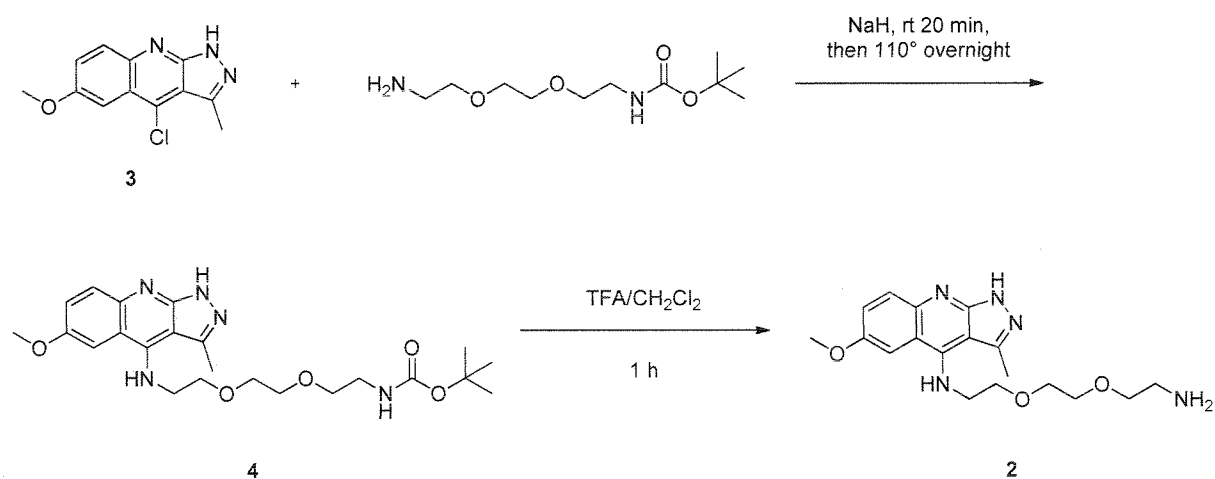


Figure 25



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Figure 26

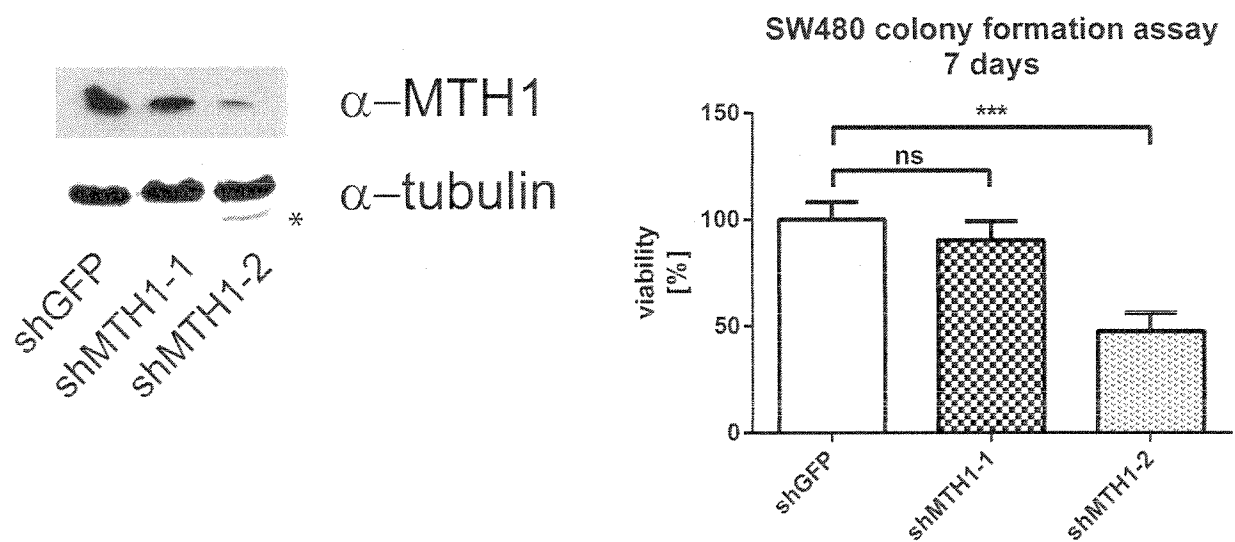


Figure 27

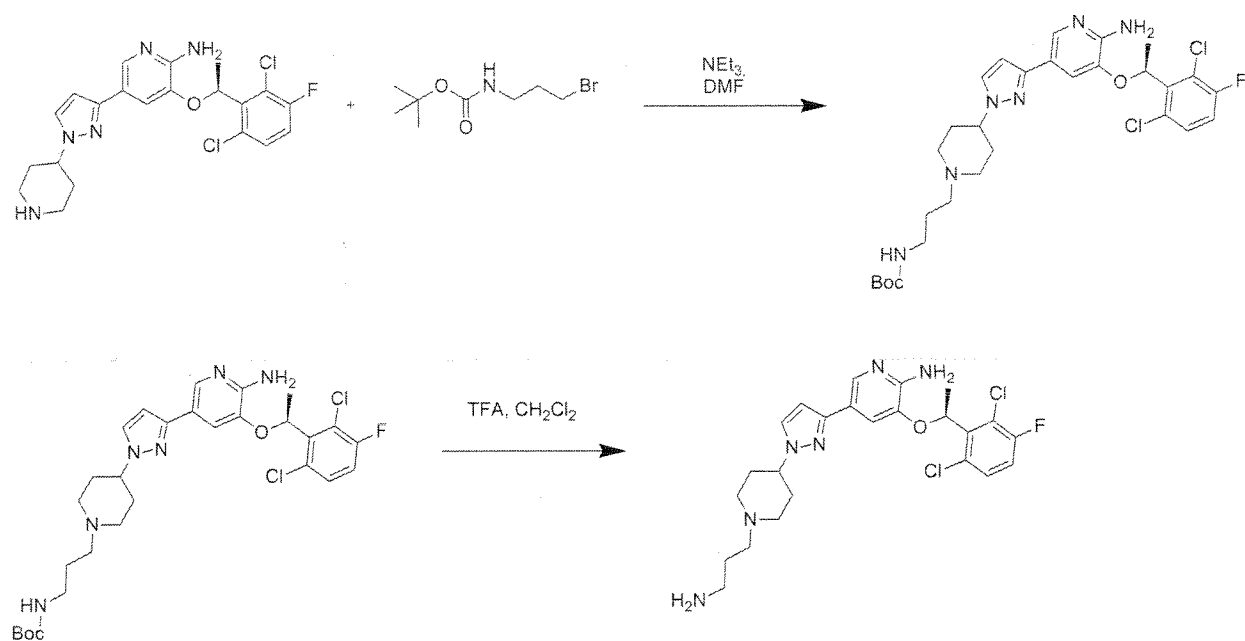


Figure 28

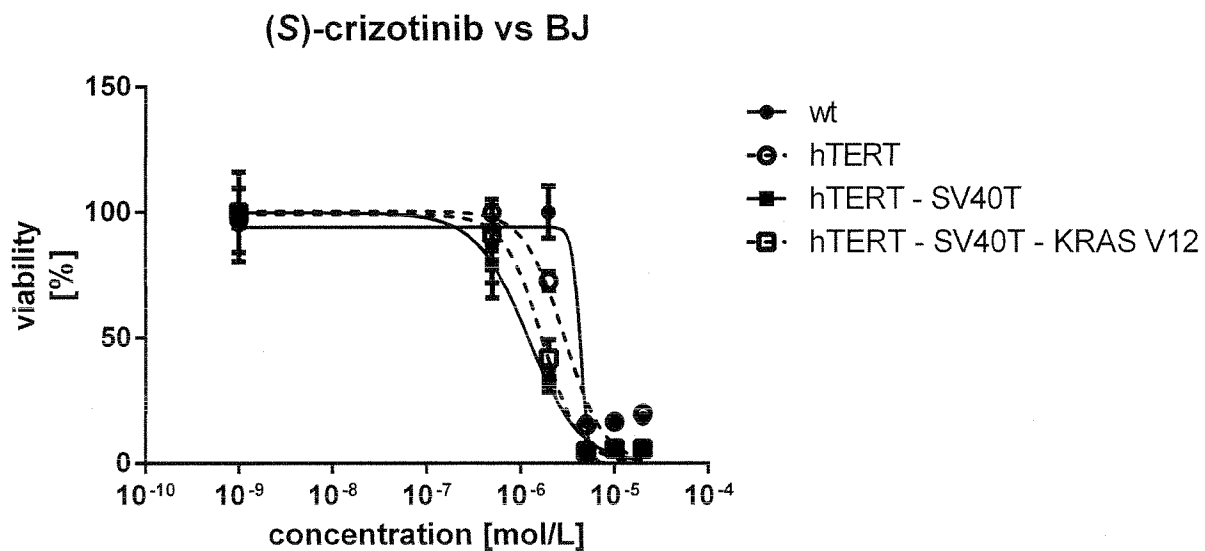


Figure 29

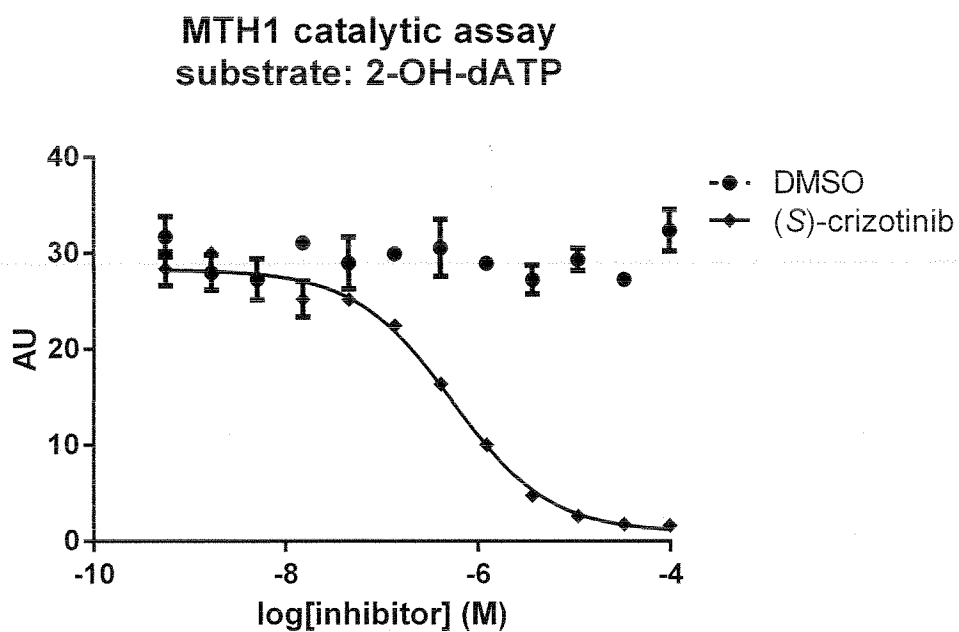
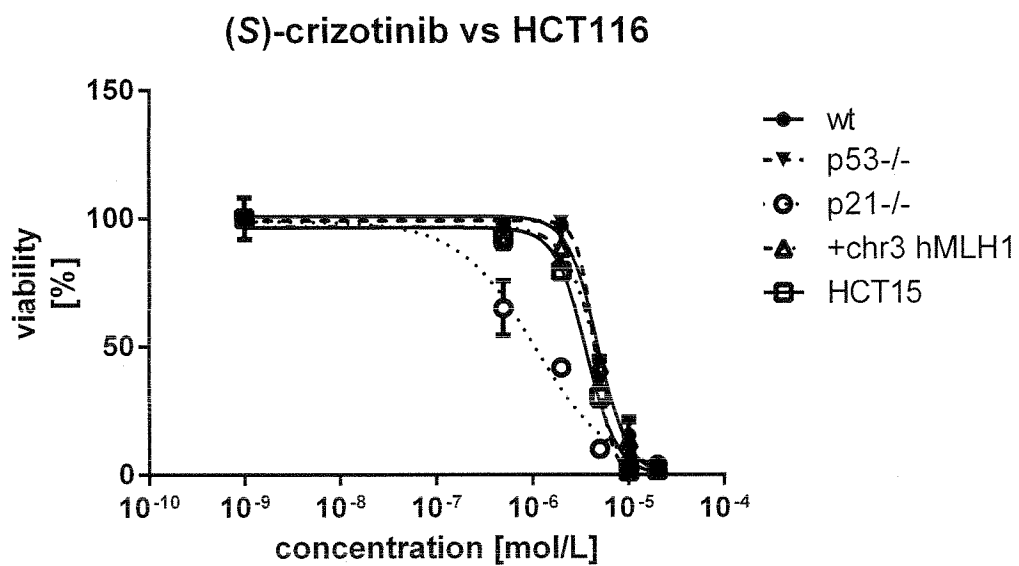


Figure 30:



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Figure 31A

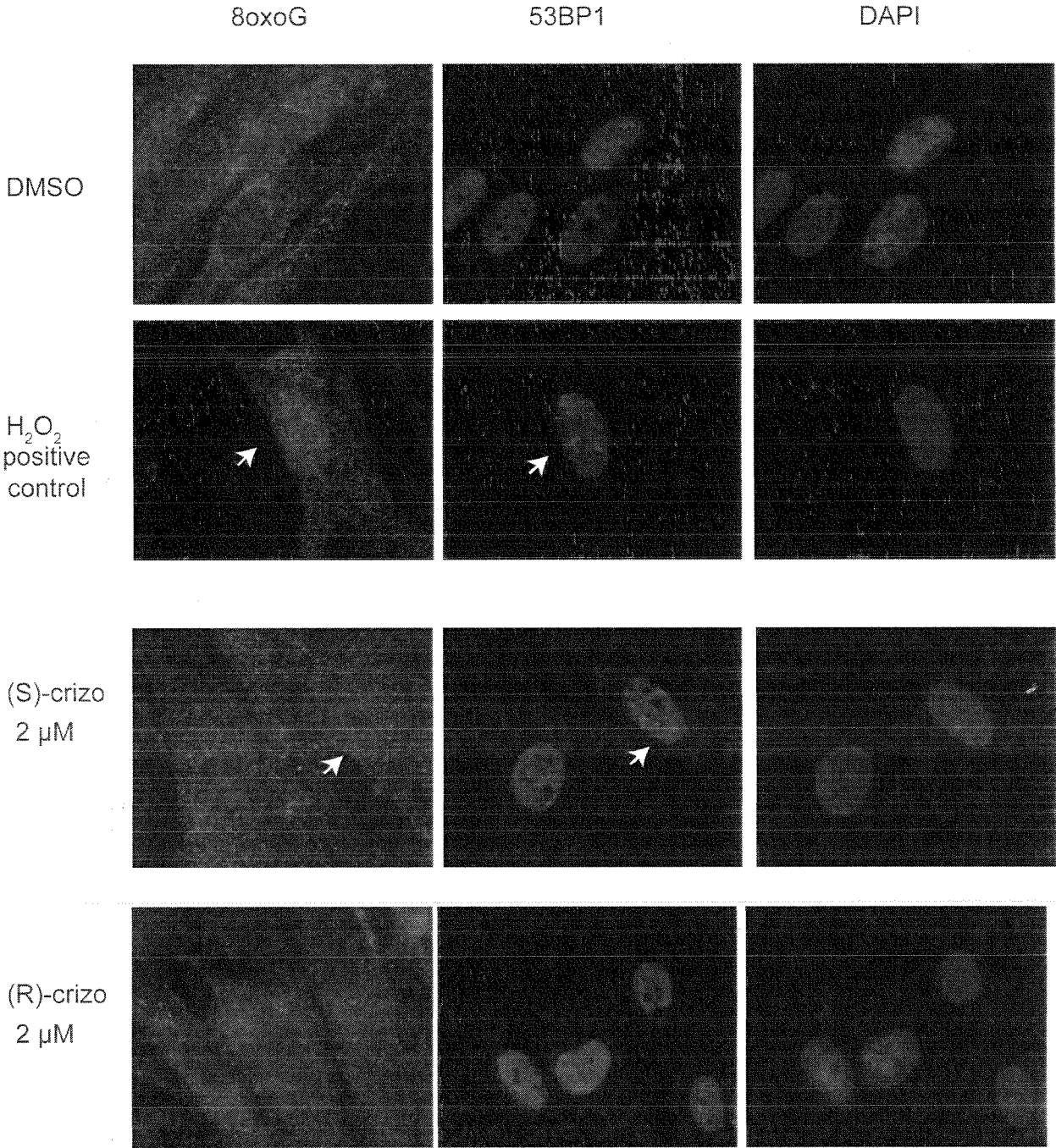


Figure 31 cont.: Figuer 31B

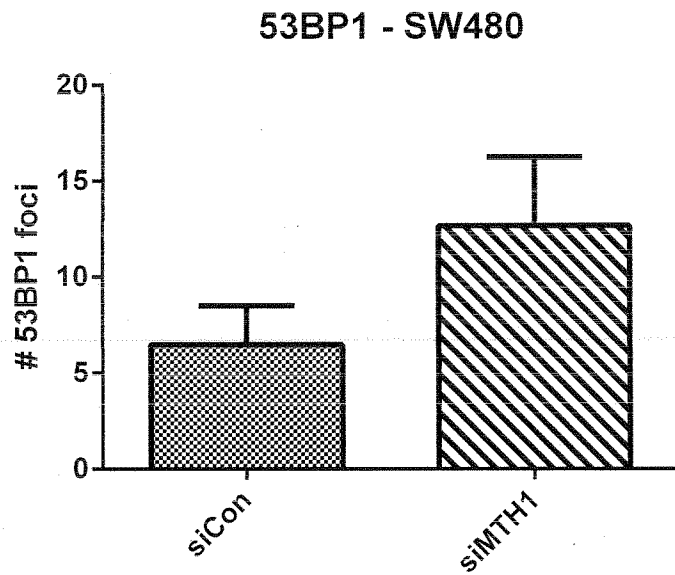
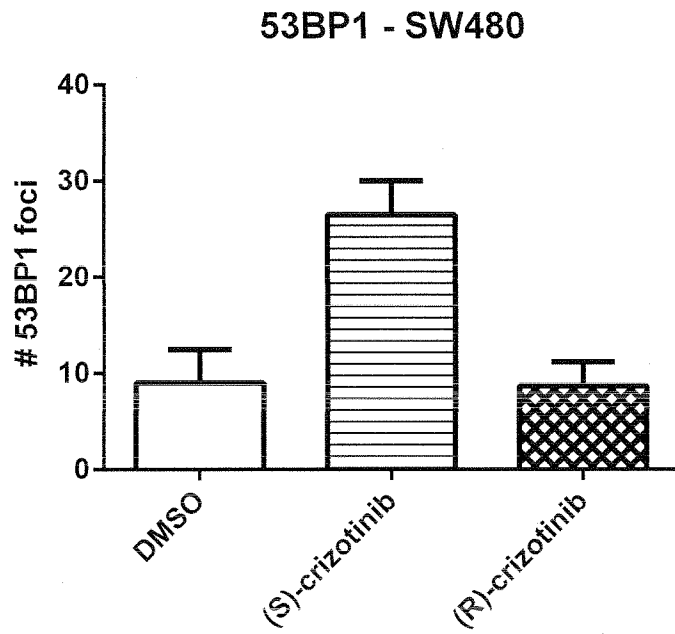
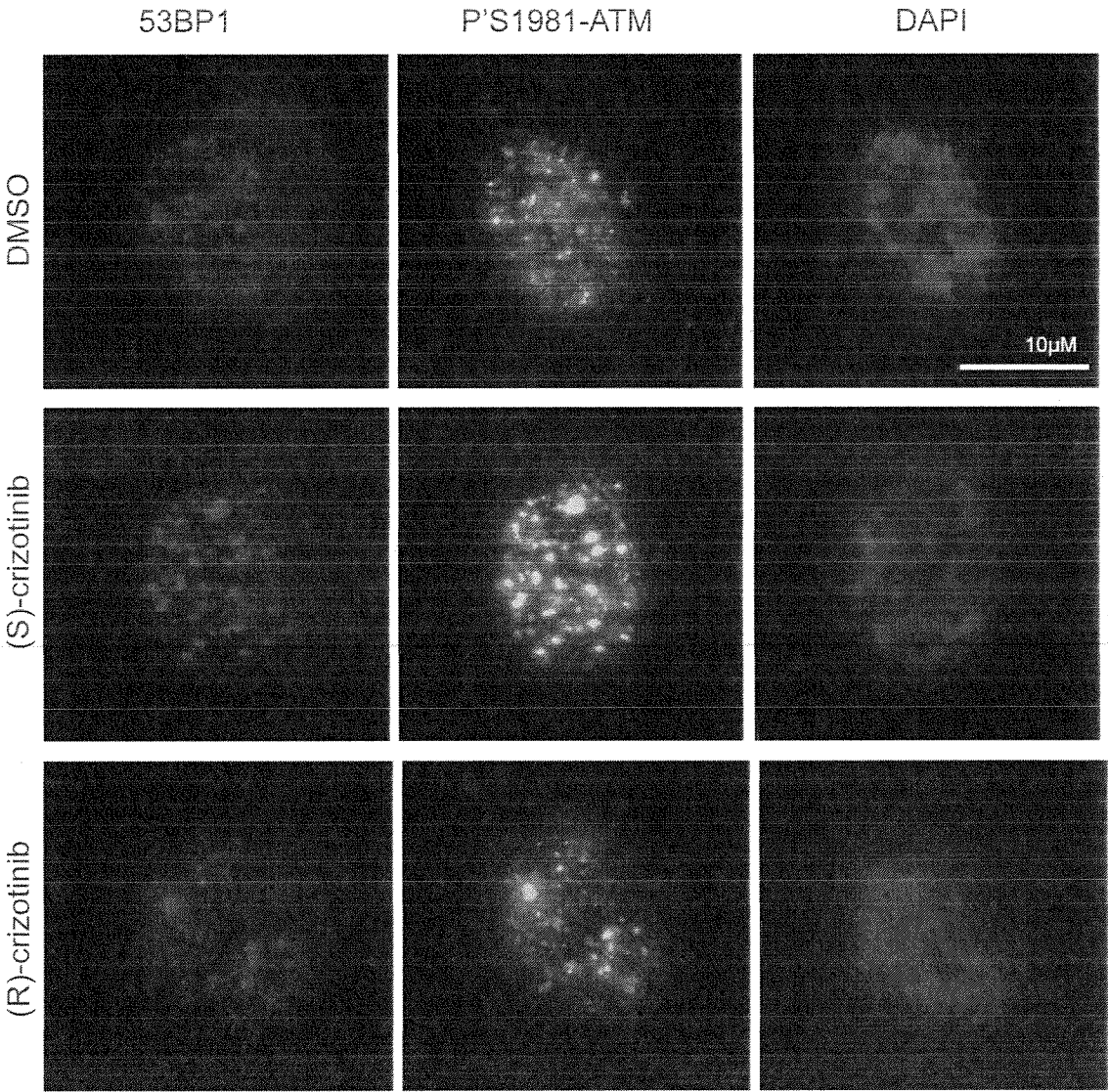
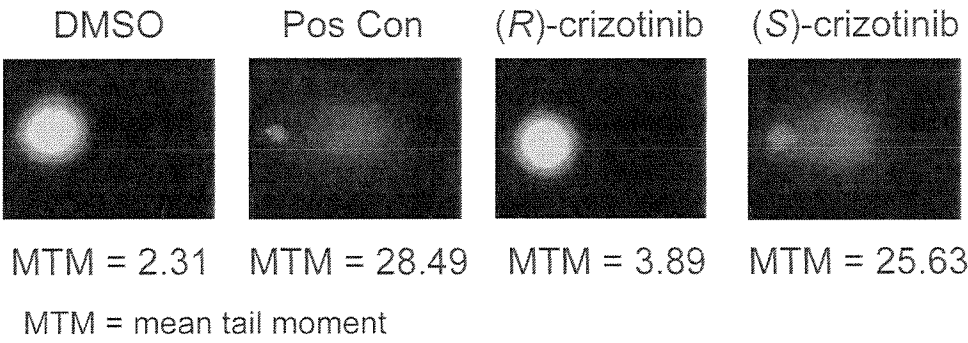


Figure 32



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/067744

A. CLASSIFICATION OF SUBJECT MATTER
 INV. A61K31/4545 A61P35/00
 ADD.

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/021881 A2 (PFIZER [US]; CUI JINGRONG JEAN [US]; FUNK LEE ANDREW [US]; JIA LEI [US] 2 March 2006 (2006-03-02) cited in the application	1-25,43
Y	See the compounds defined in the claims and in the examples, in particular compounds N. 30, 53, 75, including the racemic form of "Crizotinib" and the racemic form of a compound falling among the Formula (I) of the present application (see example 75), and their use for the treatment of cancer, alone or in combination with other anticancer agents (see from page 32, line 15 to page 36, line 35) ----- -/--	1-43



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

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Date of the actual completion of the international search

13 November 2013

Date of mailing of the international search report

19/11/2013

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

Veronese, Andrea

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/067744

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CUI J JEAN ET AL: "Structure based drug design of crizotinib (PF-02341066), a potent and selective dual inhibitor of mesenchymal-epithelial transition factor (c-MET) kinase and anaplastic lymphoma kinase (ALK).", JOURNAL OF MEDICINAL CHEMISTRY 22 SEP 2011, vol. 54, no. 18, 22 September 2011 (2011-09-22), pages 6342-6363, XP002688056, ISSN: 1520-4804 Crizotinib as an effective tumor inhibitor, and its use for the treatment of human cancer (see abstract, page 6343, left hand column, page 6354, left hand column) -----	1-25,43
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