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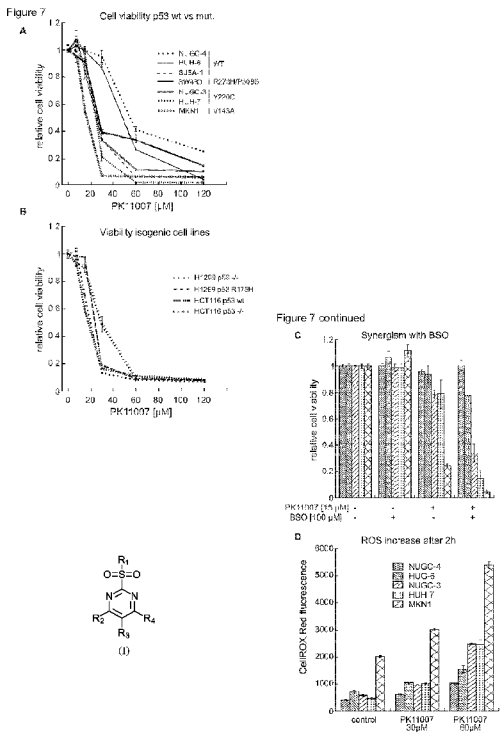
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(54) Title: 2-SULFONYLPYRIMIDINES



(I)

(57) Abstract: The invention relates to 2-sulfonylpyrimidine compounds and salts and solvates thereof for use in the treatment of a proliferative disease such as cancers. The 2- sulfonyl-primidine compounds may be administered, either simultaneously or sequentially, with one or more pharmacologically active compounds and salts and solvates thereof such as an inhibitor of glutamate cysteine ligase. The 2- sulfonylpyrimidine compound may be represented by formula (I): (I) wherein: R_1 is selected from C_{1-6} alkyl, C_{6-12} aryl, C_{7-18} aralkyl, 6- to 15-membered heteroaralkyl and 6- to 12-membered heterocyclylalkyl wherein each of these groups are optionally substituted with from one to three optional substituents; R_2 is selected from CF_3, O-C(O)-R_6, C(O)R_7, C(O)NHR_7 and NHC(O)R_7; R_3 is selected from H, F, CI, Br, I, OH, C_{1-6} alkyl, OC_{1-6} alkyl, CH_2F, CHF_2, CF_3, CN, NO_2, CO_2R_8, C(O)NHR_{10}, NHC(O)R_{10}, $(CH_2)_z$-NR_8R_9 and $(CH_2)_z$-NH-C(=NH)-NH_2; R_4 is selected from H, C_{1-6} alkyl and CH_2R_{11}; R_5 is selected from C_{1-6} alkyl; R_6 is selected from H and C_{1-6} alkyl; R_7 is selected from 5 to 9-membered heteroaryl groups, and phenyl wherein these groups are optionally substituted with from one to three optional substituents; and wherein the heteroaryl group is attached to the rest of compound of formula (I) by a carbon ring atom; R_8 and R_9 are independently selected from H, C_{1-6} alkyl and benzyl; R_{10} is selected from 5- to 9-membered heteroaryl groups, 5- and 6-membered heterocyclyl, and phenyl wherein these groups are optionally substituted with from one to three optional substituents; R_{11} is phenyl optionally substituted; and z is selected from an integer selected from o to 6.

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2-SULFONYLPYRIMIDINES

FIELD OF THE INVENTION

The invention is in the field of treatment of proliferative diseases such as cancer. In particular, the invention relates to 2-sulfonylpyrimidine compounds and salts and solvates thereof for use in the treatment of a proliferative disease.

BACKGROUND TO THE INVENTION

In nature, a wide range of posttranslational protein modifications regulate protein activity and significantly increase the diversity of protein structure and function (1, 2). Studying the functional and structural implications of covalent protein modifications is challenging because of the limited availability of pure, selectively modified proteins (1). Various chemical reactions have been developed to achieve selective modification of cysteines, lysines, N-terminal amino groups and artificially incorporated unnatural amino acids. This toolkit offers unique opportunities in chemical biology to study natural systems, create therapeutic conjugates, and generate novel protein constructs (2, 3). Because of its relatively high nucleophilicity, cysteine has a prominent position as a chemical handle in proteins and can be selectively modified over other nucleophilic residues by various electrophiles, such as maleimides, iodoacetamides, α-halocarbonyls, Michael acceptors, activated thiols, and methane/phenyl-thiosulfonates (2). Selective modification of cysteines is also therapeutically relevant, as previously reported for thiol alkylating compounds that lead to reactivation of mutant p53 in biological systems (4, 5).

The tumour suppressor p53 plays a key role in regulating cell cycle arrest, DNA repair, apoptosis and cell senescence and is inactivated either by mutation or upregulation of proteins such as MDM2 or MDMX in virtually all human cancers (6-8). The function of p53 may be impaired by mutation of residues involved directly in DNA binding (contact mutants) or mutations elsewhere in the DNA-binding domain that destabilize it (structural mutants) (9). The destabilized p53 cancer mutant Y220C can be reactivated both *in vitro* and in cancer cells by small molecules that bind to a mutationally induced crevice on the surface of the protein (10, 11). The thiol alkylating agents PRIMA-1 and APR-246, also known as PRIMA-1^{MET}, preferentially induce growth suppression and apoptosis in cancer cells harbouring either contact or destabilized mutants of p53 (5), suggesting a more general mode of mutant reactivation. Both compounds decompose into the bioactive methylene quinuclidinone (MQ), which contains a Michael acceptor group that reacts with nucleophilic thiols through a 1,4-addition. For a number of other

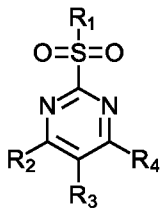
thiol reactive compounds, such as the maleimide MIRA-3, or STIMA-1 (2-vinylquinazolin-4-(3H)-one), very similar biological effects were observed (12, 13). However, despite their common reactivity towards thiols, the molecular basis for the observed biological effects of these compounds in cells still remains largely unclear. Further studies are required to understand these biological effects, to improve efficacy and to identify lead substances that may reach the clinic. Hence, there remains a need for further and more effective thiol alkylating agents that induce growth suppression and apoptosis in cancer cells.

The present invention seeks to overcome problem(s) associated with the prior art.

The present inventors have discovered that certain 2-sulfonylpyrimidine molecules, such as PK11007, stabilize the DNA-binding domain of both wild-type (WT) and mutant p53 proteins by covalent cysteine modification. The thiol reactivity and mechanism of p53 stabilization of these 2-sulfonylpyrimidines was analysed and their effects in different cancer cell lines were analysed. These 2-sulfonylpyrimidines were found to induce cell death by increasing cellular reactive oxygen species (ROS) levels.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides the present invention provides a 2-sulfonylpyrimidine compound of formula (I):



(I)

and salts and solvates thereof for use in the treatment of a proliferative disease;

wherein:

R_1 is selected from C_{1-6} alkyl, C_{6-12} aryl, C_{7-18} aralkyl, 6- to 15-membered heteroaralkyl and 6- to 12-membered heterocyclylalkyl wherein each of these groups are optionally substituted with from one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, CN, NO_2, OH, CO_2H, $CONH_2$, $C(O)NH(C_{1-6}$ alkyl), F, Cl, Br and I;

R_2 is selected from CF_3, $O\text{-}C(O)\text{-}R_6$, $C(O)R_7$, $C(O)NHR_7$ and $NHC(O)R_7$;

R_3 is selected from H, F, Cl, Br, I, OH, C_{1-6} alkyl, OC_{1-6} alkyl, CH_2F, CHF_2, CF_3, CN, NO_2, CO_2R_8, $C(O)NHR_{10}$, $NHC(O)R_{10}$, $(CH_2)_z\text{-}NR_8R_9$ and $(CH_2)_z\text{-}NH\text{-}C(=NH)\text{-}NH_2$;

R_4 is selected from H, C_{1-6} alkyl and CH_2R_{11};

R_5 is selected from C_{1-6} alkyl;

R_6 is selected from H and C_{1-6} alkyl;

R_7 is selected from 5- to 9-membered heteroaryl groups and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, SR_5, CO_2R_5, F, Cl, Br, I, NO_2, OH, benzyl, fluorobenzyl and difluorobenzyl; and wherein the heteroaryl group is attached to the rest of compound of formula (I) by a carbon ring atom;

R_8 and R_9 are independently selected from H, C_{1-6} alkyl and benzyl;

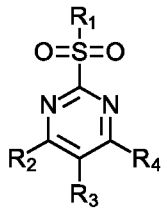
R_{10} is selected from 5- to 9-membered heteroaryl groups and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, NO_2, OH, benzyl, fluorobenzyl and difluorobenzyl;

R_{11} is phenyl optionally substituted with from 1 to 3 optional substituents independently selected from $NR_{12}R_{13}$ and OR_{12};

R_{12} and R_{13} are independently selected from H and C_{1-6} alkyl; and

z is selected from an integer selected from 0 to 6.

In a further aspect, the present invention provides a 2-sulfonylpyrimidine compound of formula (I):



(I)

and salts and solvates thereof for use in the treatment of a proliferative disease; wherein:

R_1 is selected from C_{1-6} alkyl, C_{6-12} aryl, C_{7-18} aralkyl, 6- to 15-membered heteroaralkyl and 6- to 12-membered heterocyclylalkyl wherein each of these groups are optionally substituted with from one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, CN, NO_2, OH, CO_2H, $CONH_2$, $C(O)NH(C_{1-6}$ alkyl), F, Cl, Br and I;

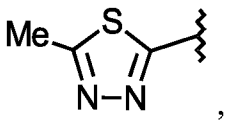
R_2 is selected from CF_3, O-C(O)-R_6, $C(O)R_7$, $C(O)NHR_7$ and $NHC(O)R_7$;

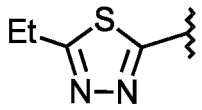
R_3 is selected from H, F, Cl, Br, I, OH, C_{1-6} alkyl, OC_{1-6} alkyl, CH_2F, CHF_2, CF_3, CN, NO_2, CO_2R_8, $C(O)NHR_{10}$, $NHC(O)R_{10}$, $(CH_2)_z$-NR_8R_9 and $(CH_2)_z$-NH-C(=NH)-NH_2;

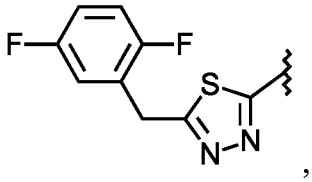
R_4 is selected from H, C_{1-6} alkyl and CH_2R_{11};

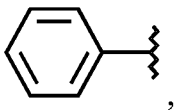
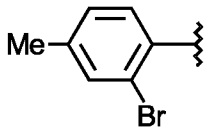
R_6 is selected from H and C_{1-6} alkyl;

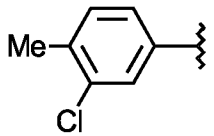
R_7 is selected from:



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and


;

R_8 and R_9 are independently selected from H, C_{1-6} alkyl and benzyl;

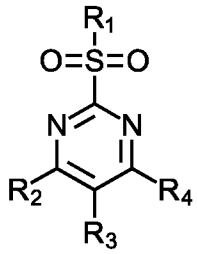
R_{10} is selected from 5- to 9-membered heteroaryl groups and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, NO_2, OH, benzyl, fluorobenzyl and difluorobenzyl;

R_{11} is phenyl optionally substituted with from 1 to 3 optional substituents independently selected from $NR_{12}R_{13}$ and OR_{12};

R_{12} and R_{13} are independently selected from H and C_{1-6} alkyl; and

z is selected from an integer selected from 0 to 6.

There is also described a 2-sulfonylpyrimidine compound of formula (I):



(I)

and salts and solvates thereof for use in the treatment of a proliferative disease;

wherein:

R_1 is selected from R_5 and NH-$(CH_2)_x$-R_5;

R_2 is selected from H, C_{1-6} alkyl, CF_3, CO_2R_6, O-C(O)-R_6, C(O)R_7, C(O)NHR_6, C(O)NHR_7, NHC(O)R_7, $(CH_2)_y$-NHR_6, $(CH_2)_y$-NR_6R_7 and $(CH_2)_y$-NH-C(=NH)-NH_2;

R_3 is selected from H, F, Cl, Br, I, OH, C_{1-6} alkyl, OC_{1-6} alkyl, CH_2F, CHF_2, CF_3, CN, NO_2, CO_2R_8, C(O)NHR_{10}, NHC(O)R_{10}, $(CH_2)_z$-NR_8R_9 and $(CH_2)_z$-NH-C(=NH)-NH_2;

R_4 is selected from H, C_{1-6} alkyl and CH_2R_{11};

R_5 is selected from C_{1-6} alkyl, C_{6-12} aryl, 5- to 9-membered heteroaryl, 5- to 6-membered heterocyclyl, C_{7-18} aralkyl, 6- to 15-membered heteroaralkyl and 6- to 12-membered heterocyclylalkyl wherein each of these groups are optionally substituted with from one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, CN, NO_2, OH, CO_2H, $CONH_2$, C(O)NH(C_{1-6} alkyl), F, Cl, Br and I;

R_6 is selected from H and C_{1-6} alkyl;
R_7 and R_{10} are independently selected from 5- to 9-membered heteroaryl groups, 5- to 6-membered heterocyclyl and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, NO_2, OH, benzyl, fluorobenzyl and difluorobenzyl;
R_8 and R_9 are independently selected from H, C_{1-6} alkyl and benzyl;
R_{11} is phenyl optionally substituted with from 1 to 3 optional substituents independently selected from $NR_{12}R_{13}$ and OR_{12};
R_{12} and R_{13} are independently selected from H and C_{1-6} alkyl;
x, y and z are independently selected from an integer selected from 0 to 6; and
at least one of R_2, R_3 and R_4 is not H.

In a further aspect, there is provided a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof for use in the treatment of a proliferative disease, wherein the 2-sulfonylpyrimidine compound is administered, either simultaneously or sequentially, with one or more pharmacologically active compounds and salts and solvates thereof.

In a further aspect, there is provided a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any one of claims 1 to 12, for use in the treatment of a proliferative disease, wherein the 2-sulfonylpyrimidine compound is administered, either simultaneously or sequentially, with one or more pharmacologically active compounds and salts and solvates thereof suitable for treating anti-proliferative diseases selected from alkylating agents, platinum compounds, DNA altering compounds, microtubule modifiers, antimetabolites, anticancer antibodies, small molecule kinase inhibitors, drug conjugates, miscellaneous antitumor agents and mixtures thereof.

In a further aspect, there is provided a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof for use in the treatment of a proliferative disease, wherein the 2-sulfonylpyrimidine compound is administered, either simultaneously or sequentially, with an inhibitor of glutamate cysteine ligase.

In a further aspect, there is provided a pharmaceutical composition comprising a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof and one or more pharmacologically active compounds and salts and solvates thereof.

In a further aspect, there is provided a pharmaceutical composition comprising a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof and an inhibitor of glutamate cysteine ligase.

In a further aspect, there is provided a pharmaceutical composition comprising a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof and an inhibitor of glutamate cysteine ligase and a pharmaceutically acceptable carrier or diluent for use in the treatment of a proliferative disease.

In a further aspect, there is provided a kit comprising: a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof and one or more pharmacologically active compounds and salts and solvates thereof.

In a further aspect, there is provided a kit comprising: a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof and an inhibitor of glutamate cysteine ligase.

In a further aspect, the present invention provides the use of a compound of formula (I) and salts and solvates thereof in the manufacture of a medicament for treating a proliferative disease.

In a further aspect, the present invention provides a method of treatment of a subject suffering from a proliferative disease, comprising administering to said subject a therapeutically effective amount of a compound of formula (I) and salts and solvates thereof or a pharmaceutical composition of the present invention.

In a further aspect, the present invention provides a method of treatment of a subject suffering from a proliferative disease, comprising administering to said subject a therapeutically effective amount of a compound of formula (I) and salts and solvates thereof, and a therapeutically effective amount of one or more pharmacologically active compounds and salts and solvates thereof or a therapeutically effective amount of a pharmaceutical composition of the present invention.

Definitions

The following abbreviations are used throughout the specification: Bn benzyl; BSO buthionine sulfoximine; Et ethyl; GSH Glutathione; Me methyl; Ph phenyl; ROS reactive oxygen species and WT wild-type.

"Substituted", when used in connection with a chemical substituent or moiety (e.g., an alkyl group), means that one or more hydrogen atoms of the substituent or moiety have been replaced with one or more non-hydrogen atoms or groups, provided that valence requirements are met and that a chemically stable compound results from the substitution.

"Optionally substituted" refers to a parent group which may be unsubstituted or which may be substituted with one or more substituents. Suitably, unless otherwise specified, when optional substituents are present the optional substituted parent group comprises from one to three optional substituents, i.e. 1, 2 or 3 optional substituents. Where a group may be optionally substituted with up to with up to three groups, this means that the group may be substituted with 0, 1, 2 or 3 of the optional substituents.

"Independently selected" is used in the context of statement that, for example, "R_8 and R_{10} are independently selected from H, C_{1-12} alkyl, etc." and means that each instance of the functional group, e.g. R_8, is selected from the listed options independently of any other instance of R_8 or R_{10} in the compound. Hence, for example, a C_{1-12} alkyl may be selected for the first instance of R in the compound and a C_{2-12} alkenyl may be selected for the next instance of R in the compound.

C_{1-6} alkyl: refers to straight chain and branched saturated hydrocarbon groups, generally having from 1 to 6 carbon atoms; more suitably C_{1-5} alkyl; more suitably C_{1-4} alkyl. Examples of alkyl groups include methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl, i-butyl, t-butyl, pent-1-yl, pent-2-yl, pent-3-yl, 3-methylbut-1-yl, 3-methylbut-2-yl, 2-methylbut-2-yl, 2,2,2-trimethyleth-1-yl, n-hexyl, n-heptyl, and the like.

"Alkylene" refers to a divalent radical derived from an alkane which may be a straight chain or branched, as exemplified by $-CH_2CH_2CH_2CH_2-$.

"Aryl": refers to fully unsaturated monocyclic, bicyclic and polycyclic aromatic hydrocarbons having at least one aromatic ring and having a specified number of carbon atoms that comprise their ring members (e.g., C_{6-12} aryl refers to an aryl group having 6 to 12 carbon atoms as ring members). The aryl group may be attached to a parent group or to a substrate at any ring atom and may include one or more non-hydrogen substituents unless such attachment or substitution would violate valence requirements. Examples of aryl groups include phenyl, biphenyl and naphthaleneyl.

"C_{7-18} aralkyl" refers to an arylalkyl group having 7 to 18 carbon atoms and comprising an alkyl group substituted with an aryl group. Hence, the aralkyl group is attached to the rest of the molecule at a carbon of the alkyl group. Suitably the alkyl group is a C_{1-6} alkyl group and the aryl group is a C_{6-12} aryl group, such as phenyl. Examples of C_{7-18} aralkyl include benzyl and phenethyl. In some cases the C_{7-18} aralkyl group may be optionally substituted and an example of an optionally substituted C_{7-18} aralkyl group is 4-methoxylbenzyl.

"5- to 9-membered ring heteroaryl": refers to unsaturated monocyclic or bicyclic aromatic groups comprising 5 to 9 ring atoms, whether carbon or heteroatoms, of which from 1 to 5 are ring heteroatoms. Suitably, any monocyclic heteroaryl ring has from 5 to 6 ring atoms including from 1 to 3 ring heteroatoms. Suitably each ring heteroatom is independently selected from nitrogen, oxygen, and sulfur. The bicyclic rings include fused ring systems and, in particular, include bicyclic groups in which a monocyclic heterocycle comprising 5 ring atoms is fused to a benzene ring. The heteroaryl group may be attached to a parent group or to a substrate at any ring atom and may include one or more non-hydrogen substituents unless such attachment or substitution would violate valence requirements or result in a chemically unstable compound.

Examples of monocyclic heteroaryl groups include, but are not limited to, those derived from:

N_1: pyrrole, pyridine;
O_1: furan;
S_1: thiophene isoxazole, isoxazine;
N_1O_1: oxazole, isoxazole;
N_2O_1: oxadiazole (e.g. 1-oxa-2,3-diazolyl, 1-oxa-2,4-diazolyl, 1-oxa-2,5-diazolyl, 1-oxa-3,4-diazolyl);
N_3O_1: oxatriazole;
N_1S_1: thiazole, isothiazole;
N_2S_1: thiadiazole (e.g. 1,3,4-thiadiazole);
N_2: imidazole, pyrazole, pyridazine, pyrimidine, pyrazine;
N_3: triazole, triazine; and,
N_4: tetrazole.

Examples of heteroaryl which comprise fused rings, include, but are not limited to, those derived from:

O_1: benzofuran, isobenzofuran;

N_1: indole, isoindole, indolizine, isoindoline;

S_1: benzothiofuran;

N_1O_1: benzoxazole, benzisoxazole;

N_1S_1: benzothiazole;

N_2: benzimidazole, indazole;

O_2: benzodioxole;

N_2O_1: benzofurazan;

N_2S_1: benzothiadiazole;

N_3: benzotriazole; and

N_4: purine (e.g., adenine, guanine).

"6- to 15-membered heteroarylalkyl" refers to an alkyl group substituted with a heteroaryl group. Hence, the heteroarylalkyl group is attached to the rest of the molecule at a carbon of the alkyl group. Suitably the alkyl is a C_{1-6} alkyl group and the heteroaryl group is 5- to 9-membered heteroaryl as defined above. Examples of 6- to 15-membered heteroarylalkyl groups include pyrrol-2-ylmethyl, pyrrol-3-ylmethyl, pyrrol-4-ylmethyl, pyrrol-3-ylethyl, pyrrol-4-ylethyl, imidazol-2-ylmethyl, imidazol-4-ylmethyl, imidazol-4-ylethyl, thiophen-3-ylmethyl, furan-3-ylmethyl, pyridin-2-ylmethyl, pyridin-2-ylethyl, thiazol-2-ylmethyl, thiazol-4-ylmethyl, thiazol-2-ylethyl, pyrimidin-2-ylpropyl, and the like.

"5- to 6-membered heterocyclyl": refers to saturated or partially unsaturated monocyclic groups having ring atoms composed of from 5 to 6 ring atoms, whether carbon atoms or heteroatoms, of which from 1 to 4 are ring heteroatoms. The ring heteroatoms are independently selected from nitrogen, oxygen, and sulphur.

The heterocyclyl group may be attached to a parent group or to a substrate at any ring atom and may include one or more non-hydrogen substituents unless such attachment or substitution would violate valence requirements or result in a chemically unstable compound.

Examples of monocyclic heterocyclyl groups include, but are not limited to, those derived from:

N_1 : aziridine, azetidine, pyrrolidine, pyrroline, 2H-pyrrole or 3H-pyrrole, piperidine, dihydropyridine, tetrahydropyridine, azepine;
O_1: oxirane, oxetane, tetrahydrofuran, dihydrofuran, tetrahydropyran, dihydropyran, pyran, oxepin;
S_1: thiirane, thietane, tetrahydrothiophene, tetrahydrothiopyran, thiepane;
O_2: dioxoiane, dioxane, and dioxepane;
O_3: trioxane;
N_2: imidazoiidine, pyrazolidine, imidazoline, pyrazoline, piperazine:
N_1O_1: tetrahydrooxazole, dihydrooxazole, tetrahydroisoxazole, dihydroisoxazole, morpholine, tetrahydrooxazine, dihydrooxazine, oxazine;
N_1S_1: thiazoline, thiazolidine, thiomorpholine;
N_2O_1: oxadiazine;
O_1S_1: oxathiole and oxathiane (thioxane); and
$N_1O_1S_1$: oxathiazine.

"6- to 12-membered heterocyclylalkyl" refers to an alkyl group substituted with a heterocyclyl group. Hence, the heterocyclylalkyl group is attached to the rest of the molecule at a carbon of the alkyl group. Suitably the alkyl is a C_{1-6} alkyl group and the heterocyclyl group is 5- to 6-membered heterocyclyl group as defined above.

"Drug", "drug substance", "active pharmaceutical ingredient", and the like, refer to a compound (e.g., compounds of Formula 1 and compounds specifically named above) that may be used for treating a subject in need of treatment.

"Excipient" refers to any substance that may influence the bioavailability of a drug, but is otherwise pharmacologically inactive.

"Pharmaceutically acceptable" substances refers to those substances which are within the scope of sound medical judgment suitable for use in contact with the tissues of subjects without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit-to-risk ratio, and effective for their intended use.

"Pharmaceutical composition" refers to the combination of one or more drug substances and one or more excipients.

The term "subject" as used herein refers to a human or non-human mammal. Examples of non-human mammals include livestock animals such as sheep, horses, cows, pigs, goats, rabbits and deer; and companion animals such as cats, dogs, rodents, and horses.

"Therapeutically effective amount" of a drug refers to the quantity of the drug or composition that is effective in treating a subject and thus producing the desired therapeutic, ameliorative, inhibitory or preventative effect. The therapeutically effective amount may depend on the weight and age of the subject and the route of administration, among other things.

"Treating" refers to reversing, alleviating, inhibiting the progress of, or preventing a disorder, disease or condition to which such term applies, or to reversing, alleviating, inhibiting the progress of, or preventing one or more symptoms of such disorder, disease or condition.

"Treatment" refers to the act of "treating", as defined immediately above.

As used herein the term "comprising" means "consisting at least in part of". When interpreting each statement in this specification that includes the term "comprising", features other than that or those prefaced by the term may also be present. Related terms such as "comprise" and "comprises" are to be interpreted in the same manner.

R_1

Suitably R_1 is selected from C_{1-6} alkyl, phenyl, C_{7-12} aralkyl, 6- to 12-membered heteroaralkyl, 6- to 12-membered heterocyclylalkyl wherein each of these groups are optionally substituted with from one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, CN, NO_2, OH, CO_2H, $CONH_2$, $C(O)NH(C_{1-6}$ alkyl), F, Cl, Br and I.

Suitably R_1 is selected from C_{1-6} alkyl; phenyl; and C_{7-12} aralkyl comprising a C_{1-6} alkyl group substituted with phenyl; wherein each of these groups are optionally substituted with from one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, CN, NO_2, OH, CO_2H, $CONH_2$, $C(O)NH(C_{1-6}$ alkyl), F, Cl, Br and I.

Suitably R_1 is selected from methyl, ethyl, phenyl and benzyl; optionally substituted with from one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, CN, NO_2, OH, CO_2H, $CONH_2$, $C(O)NH(C_{1-6}$ alkyl), F, Cl, Br and I.

Suitably the R_1 group is optionally substituted with one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, F, Cl, Br and I.

Suitably the R_1 group is optionally substituted with one, two or three optional substituents independently selected from F, Cl, Br and I.

Suitably R_1 is selected from methyl, ethyl, phenyl, benzyl, para-fluorobenzyl and ortho-fluorobenzyl.

Suitably R_1 is selected from methyl, ethyl and para-fluorobenzyl.

R_2

Suitably R_2 is selected from CF_3, $C(O)R_7$, $C(O)NHR_7$ and $NHC(O)R_7$,.

More suitably R_2 is $C(O)NHR_7$.

R_3

Suitably R_3 is selected from H, F, Cl, Br, I, OH, methyl, ethyl, OCH_3, OCH_2CH_3, CH_2F, CHF_2, CF_3, CN, NO_2, CO_2R_8, $C(O)NHR_{10}$, $NHC(O)R_{10}$, $(CH_2)_z$-NR_8R_9, and $(CH_2)_z$-NH-C(=NH)-NH_2.

Suitably R_3 is selected from H, F, Cl, Br, I, OH, methyl, ethyl, OCH_3, OCH_2CH_3, CH_2F, CHF_2, CF_3, CN, NO_2, CO_2H and $(CH_2)_z$-NR_8R_9.

Suitably R_3 is selected from H, F, Cl, Br, I, OH, $N(CH_3)_2$ and $N(CH_2Ph)_2$.

Suitably R_3 is selected from H, Cl, Br, OH and $N(CH_2Ph)_2$.

Suitably R_3 is selected from H, Cl and Br.

More suitably, R_3 is selected from Cl and Br.

R_4

Suitably R_4 is selected from H, methyl, ethyl, propyl, butyl and CH_2R_{11}.

Suitably R_4 is selected from H, methyl and CH_2R_{11}.

More suitably R_4 is H.

R_5

Suitably R_5 is selected from methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl, i-butyl and t-butyl.

More suitably R_5 is selected from methyl, ethyl, n-propyl and i-propyl .

R_6

Suitably R_6 is selected from H, methyl, ethyl, propyl and butyl.

Suitably R_6 is selected from H, methyl and ethyl.

More suitably R_6 is selected from H and methyl.

R_7

R_7 is selected from 5- to 6-membered heteroaryl groups and phenyl wherein these groups are optionally substituted with from one, two or three optional substituents independently selected from C_{1-6} alkyl, SR_5, CO_2R_5, F, Cl, Br, I, OH, benzyl, fluorobenzyl and difluorobenzyl; and wherein the heteroaryl group is attached to the rest of compound of formula (I) by a carbon ring atom. Hence, when R_7 is a heteroaryl group, it cannot be attached to the rest of compound of formula (I) by a heteroatom of the heteroaryl ring. In addition, the phrase "wherein these groups are optionally substituted" means that any selected 5-to 6-membered heteroaryl group or phenyl group may be optionally substituted with from one, two or three optional substituents selected from the list.

Suitably R_7 is selected from 5- to 6-membered heteroaryl groups and phenyl selected from pyrrolyl, pyridinyl, furanyl, thiophenyl, oxazolyl, isoxazolyl, oxadiazolyl, oxatriazolyl, thiazolyl, isothiazolyl, thiadiazolyl, imidazolyl, pyrazolyl, triazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, benzofuranyl, indolyl, benzothiofuanyl, benzothiazole, benzoxazolyl, benzimidazolyl and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently

selected from C_{1-6} alkyl, SR_5, CO_2R_5, F, Cl, Br, I, benzyl, fluorobenzyl and difluorobenzyl; and wherein the heteroaryl group is attached to the rest of compound of formula (I) by a carbon ring atom.

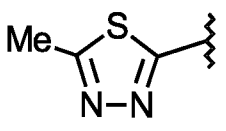
Suitably R_7 is selected from 5- to 6-membered heteroaryl groups and phenyl selected from pyrrolyl, pyridinyl, thiophenyl, oxazolyl, thiazolyl, thiadiazolyl, imidazolyl, pyrazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, benzothiazole and phenyl optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, SR_5, CO_2R_5, F, Cl, Br, I, OH, benzyl, fluorobenzyl and difluorobenzyl; and wherein the heteroaryl group is attached to the rest of compound of formula (I) by a carbon ring atom.

Suitably R_7 is selected from thiophenyl, 1,3,4-thiadiazolyl, benzothiazole and phenyl optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, SR_5, CO_2R_5, F, Cl, Br, I, benzyl, fluorobenzyl and difluorobenzyl; and wherein the thiophenyl, 1,3,4-thiadiazolyl or benzothiazole groups are attached to the rest of compound of formula (I) by a carbon ring atom.

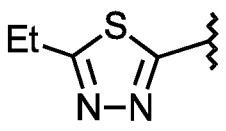
Suitably R_7 is selected from thiophenyl, 1,3,4-thiadiazolyl, benzothiazole and phenyl optionally substituted with from one to two optional substituents independently selected from methyl, ethyl, propyl, SCH_3, SCH_2CH_3, CO_2CH_3, $CO_2CH_2CH_3$, F, Cl, Br and 2,5-difluorobenzyl; and wherein the thiophenyl, 1,3,4-thiadiazolyl or benzothiazole groups are attached to the rest of compound of formula (I) by a carbon ring atom.

Suitably R_7 is selected from 1,3,4-thiadiazolyl and phenyl optionally substituted with from one to two optional substituents independently selected from methyl, ethyl, propyl, F, Cl, Br and 2,5-difluorobenzyl; and wherein the 1,3,4-thiadiazolyl group is attached to the rest of compound of formula (I) by a carbon ring atom.

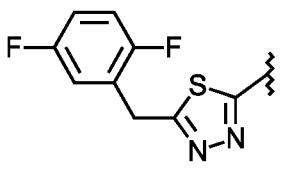
More suitably R_7 is selected from:



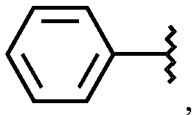
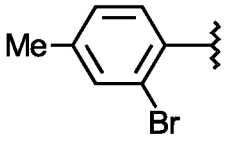
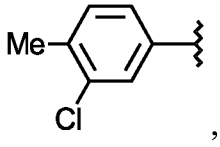
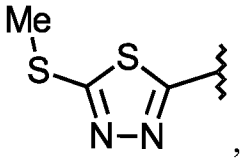
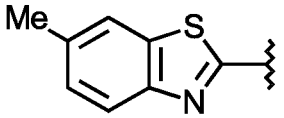
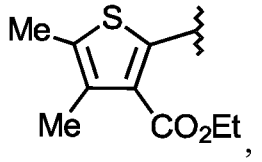
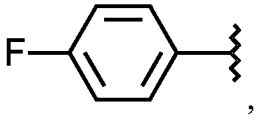
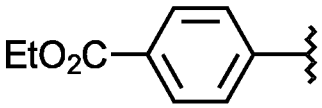
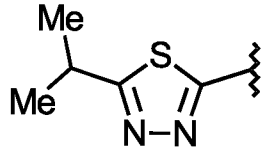
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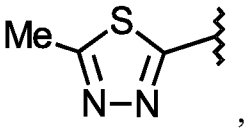
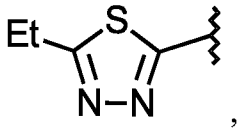
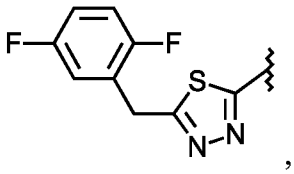
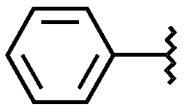
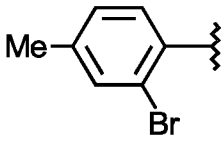
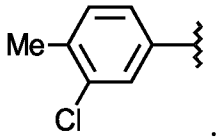


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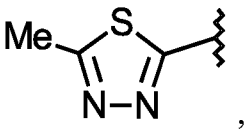
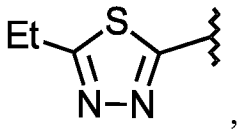
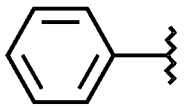

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and


The zig-zag line represent where the above R_7 groups are attached to the rest of the molecule.

More suitably R_7 is selected from:


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and

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More suitably R_7 is selected from:


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, and


R_8

Suitably R_8 is selected from H, C_{1-6} alkyl and benzyl.

Suitably R_8 is selected from H, methyl, ethyl, propyl and benzyl.

More suitably R_8 is selected from methyl and benzyl.

More suitably R_8 is benzyl.

R_9

Suitably R_9 is selected from H, C_{1-6} alkyl and benzyl.

Suitably R_9 is selected from H, methyl, ethyl, propyl and benzyl.

More suitably R_9 is selected from methyl and benzyl.

More suitably R_9 is benzyl.

R_{10}

In one embodiment, suitably R_{10} is selected from 5- to 6-membered heteroaryl, 5- to 6-membered heterocyclyl and phenyl optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, OH, benzyl, fluorobenzyl and difluorobenzyl.

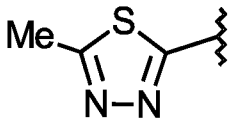
In another embodiment, suitably R_{10} is selected from pyrrolyl, pyridinyl, furanyl, thiophenyl, oxazolyl, isoxazolyl, oxadiazolyl, oxatriazolyl, thiazolyl, isothiazolyl, thiadiazolyl, imidazolyl, pyrazolyl, triazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, piperidinyl, tetrahydropyranyl, morpholinyl, pyrrolidinyl, benzofuranyl, indolyl, benzothiofuanyl, benzoxazolyl, benzimidazolyl and phenyl optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, benzyl, fluorobenzyl and difluorobenzyl.

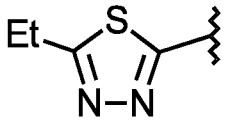
Suitably R_{10} is selected from pyrrolyl, pyridinyl, oxazolyl, thiazolyl, thiadiazolyl, imidazolyl, pyrazolyl, pyridazinyl, pyrimidinyl, pyrazinyl piperidinyl, tetrahydropyranyl, morpholinyl, pyrrolidinyl and phenyl optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, OH, benzyl, fluorobenzyl and difluorobenzyl.

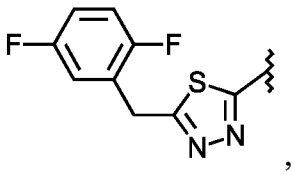
Suitably R_{10} is selected from 1,3,4-thiadiazolyl and phenyl optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, benzyl, fluorobenzyl and difluorobenzyl.

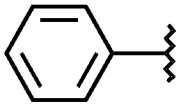
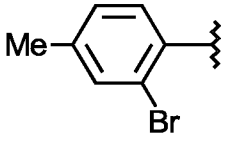
Suitably R_{10} is selected from 1,3,4-thiadiazolyl and phenyl optionally substituted with from one to two optional substituents independently selected from methyl, ethyl, propyl, F, Cl, Br and 2,5-difluorobenzyl.

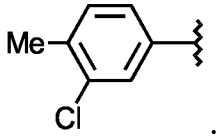
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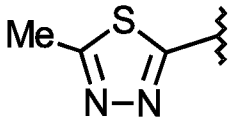
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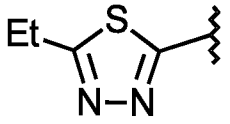
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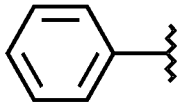
and


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More suitably R_{10} is selected from:



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, and


$\underline{R_{11}}$

Suitably R_{11} is phenyl optionally substituted with from 1 to 3 optional substituents independently selected from NH_2, $N(CH_3)_2$, OH and OCH_3 groups.

Suitably R_{11} is phenyl optionally substituted with from 1 to 3 optional OCH_3 groups.

More suitably R_{11} is 2-methoxyphenyl.

$\underline{R_{12}}$

Suitably R_{12} is selected from H, methyl, ethyl, propyl and butyl.

Suitably R_{12} is selected from H, methyl and ethyl.

More suitably R_{12} is methyl.

$\underline{R_{13}}$

Suitably R_{13} is selected from H, methyl, ethyl, propyl and butyl.

Suitably R_{13} is selected from H, methyl and ethyl.

$\underline{x}$

x is selected from an integer from 0 to 6, hence, x is selected from 0, 1, 2, 3, 4, 5 and 6.

Suitably x is selected from 0, 1, 2 and 3.

More suitably x is selected from 0 and 1.

More suitably x is 0.

y

y is selected from an integer from 0 to 6, hence, y is selected from 0, 1, 2, 3, 4, 5 and 6.

Suitably y is selected from 0, 1, 2 and 3.

More suitably y is selected from 0 and 1.

More suitably y is 0.

z

z is selected from an integer from 0 to 6, hence, z is selected from 0, 1, 2, 3, 4, 5 and 6.

Suitably z is selected from 0, 1, 2 and 3.

More suitably z is selected from 0 and 1.

More suitably z is 0.

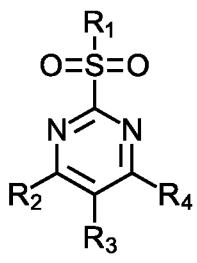
Other Issues

Suitably, at least one of R_3 and R_4 is not H.

More suitably R_3 is not H.

Further embodiments

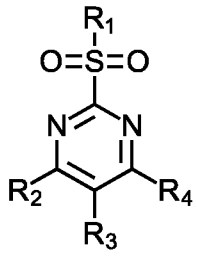
A 2-sulfonylpyrimidine compound of formula (I):



(I)

and salts and solvates thereof for use in the treatment of a proliferative disease;
wherein:
R_1 is selected from C_{1-6} alkyl, phenyl and C_{7-12} aralkyl optionally substituted with from one to three optional substituents selected from F, Cl, Br and I;
R_2 is selected from CF_3 and $C(O)NHR_7$;
R_3 is selected from H, F, Cl, Br, I, OH and $(CH_2)_z$-NR_8R_9;
z is an integer selected from 0 to 6;
R_4 is selected from H, C_{1-6} alkyl and CH_2R_{11};
R_6 is selected from H and C_{1-6} alkyl;
R_7 is selected from 5-membered heteroaryl groups and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, OH, benzyl, fluorobenzyl and difluorobenzyl; and wherein the heteroaryl group is attached to the rest of compound of formula (I) by a carbon ring atom;
R_8 and R_9 are independently selected from H, C_{1-6} alkyl and benzyl;
R_{11} is phenyl optionally substituted with from 1 to 3 optional substituents independently selected from $NR_{12}R_{13}$ and OR_{12}; and
R_{12} and R_{13} are independently selected from H and C_{1-6} alkyl.

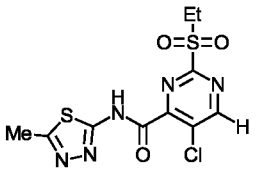
In some embodiments, the 2-sulfonylpyrimidine compound is a compound of formula (I):

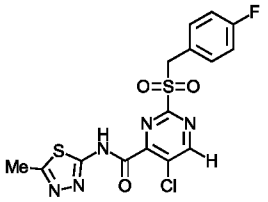


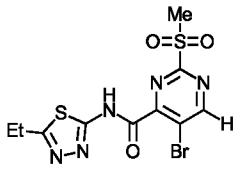
(I)

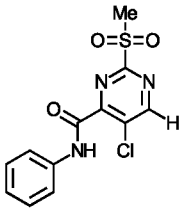
and salts and solvates thereof for use in the treatment of a proliferative disease;
wherein:
R_1 is selected from C_{1-6} alkyl and benzyl optionally substituted with from one to three optional substituents selected from F, Cl, Br and I;
R_2 is $C(O)NHR_7$;
R_3 is selected from Cl and Br;
R_4 is H;and
R_7 is 1,3,4-thiadiazolyl and phenyl substituted with from one substituents selected from C_{1-6} alkyl and 2,5-difluorobenzyl; and wherein the 1,3,4-thiadiazolyl is attached to the rest of compound of formula (I) by a carbon ring atom.

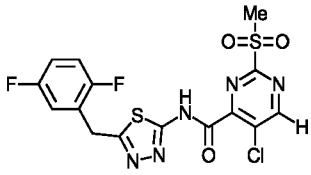
In some embodiments, the compound of formula (I) is selected from:

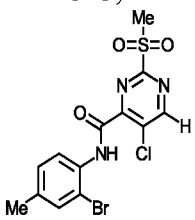

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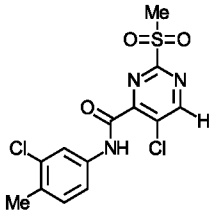

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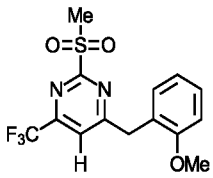

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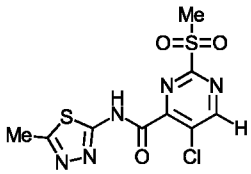

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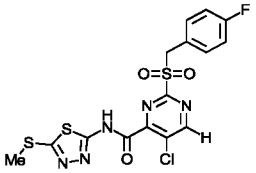

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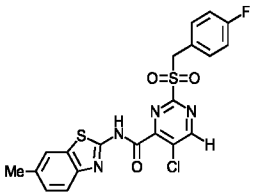

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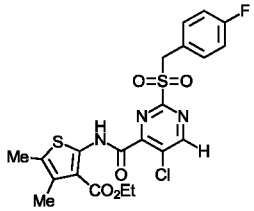

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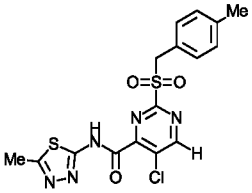

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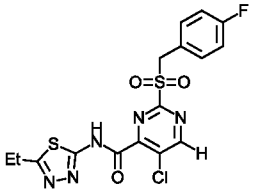

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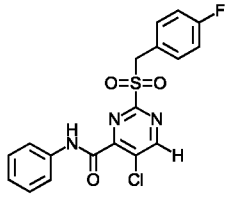

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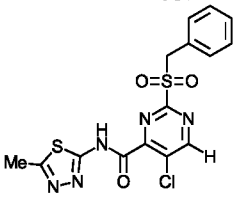

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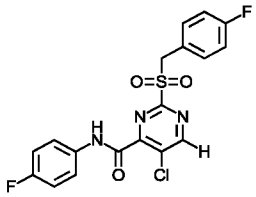

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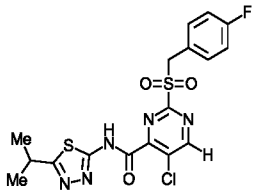

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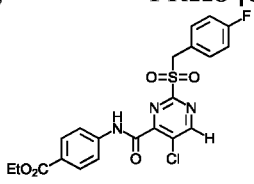

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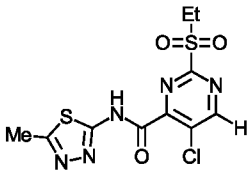
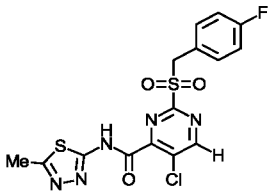
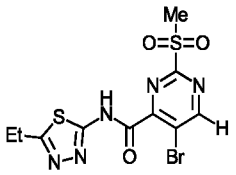
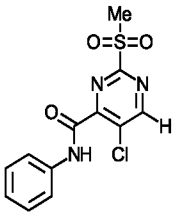
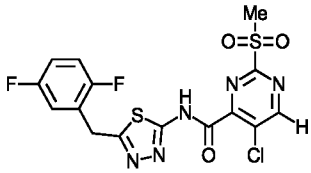
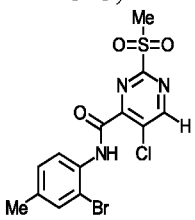
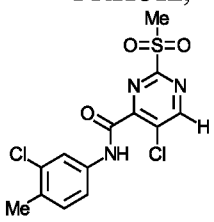
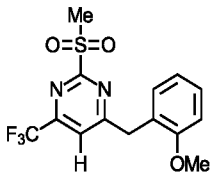
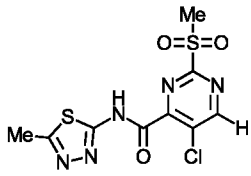

PK11048,


PK11049

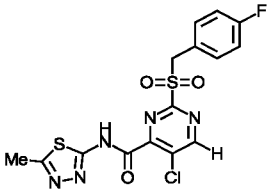
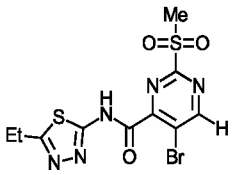
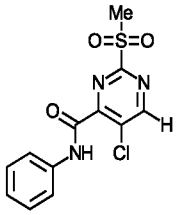
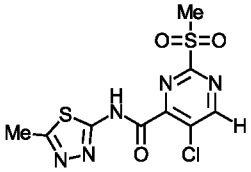
and


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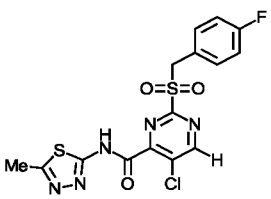
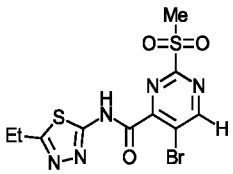
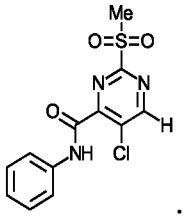
Suitably, the compound of formula (I) is selected from


PK11006,

PK11007,

PK11010,

PK11012,

PK11013,

PK11014,

PK11015,

PK11018
and

PK11029.

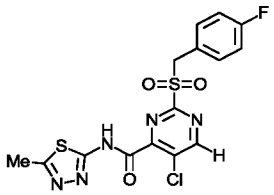
More suitably the compound of formula (I) is selected from:


PK11007
,

PK11010
,

PK11012
and

PK11029.

More suitably the compound of formula (I) is selected from:


PK11007
,

PK11010
and

PK11012
.

More suitably the compound of formula (I) is



PK11007.

Other Aspects

In some aspects the invention also involves administration of an inhibitor of glutamate cysteine ligase.

Suitably the inhibitor of glutamate cysteine ligase is selected from buthionine sulfoximine, proprothionine sulfoximine, methionine sulfoximine, ethionine sulfoximine, methyl buthionine sulfoximine, γ-glutamyl-α-aminobutyrate and γ -glutamylcysteine

More suitably the inhibitor of glutamate cysteine ligase is buthionine sulfoximine.

In some aspects the invention relates to a pharmaceutical composition. Suitably such compositions further comprise a pharmaceutically acceptable carrier or diluent.

Suitably the inhibitor of glutamate cysteine ligase is present at a concentration of from 0.1 μM to 500 μM; suitably at a concentration of from 1 μM to 300 μM; suitably at a concentration of from 10 μM to 200 μM.

Applications

The invention finds application in the treatment of proliferative diseases.

The term "proliferative disease" refers to an unwanted or uncontrolled cellular proliferation of excessive or abnormal cells which is undesired, such as, neoplastic or hyperplastic growth, whether *in vitro* or *in vivo*.

In some embodiments, the proliferative disease may be a cell proliferative disease selected from the group comprising an angiogenic disease, a metastatic disease, a tumourigenic disease, a neoplastic disease and cancer.

Any type of cell may be treated, including but not limited to bladder, bone, brain, breast (mammary), cervical, gastrointestinal (including, e.g. bowel, colon), kidney (renal), liver (hepatic), lung, ovarian, pancreas, prostate, skin, stomach and thyroid.

Examples of proliferative conditions include, but are not limited to, benign, pre-malignant, and malignant cellular proliferation, including but not limited to, neoplasms and tumours (e.g. histocytoma, glioma, astrocyoma, osteoma, osteosarcoma), cancers (e.g. adenosquamous carcinoma, anal carcinoma, bladder cancer, bone cancer [including osteosarcoma], bowel cancer, brain cancer [including glioblastoma], breast cancer [in particular, triple negative breast cancer], central nervous system (CNS) cancer, cervical cancer, colon cancer, colorectal carcinoma, Dukes' type B colorectal adenocarcinoma, endometrial or uterine carcinoma, gastric or stomach cancer [including gastrointestinal cancer and gastric adenocarcinoma] head and neck cancer, Kaposi's sarcoma, kidney or renal cancer, liver cancer [including hepatocellular cancer, hepatoma, hepatoblastoma and hepato cellular carcinoma] lung cancer [including small cell lung cancer, non-small cell lung cancer and squamous lung cancer], ovarian cancer, pancreas cancer, penile carcinoma, prostate cancer, rectal cancer, colorectal cancer, sarcoma, salivary gland carcinoma, skin cancer [including basal cell carcinoma, melanoma and squamous cell carcinoma] thyroid cancer and vulval cancer), leukemias, lymphoma, psoriasis, bone diseases, fibroproliferative disorders (e.g. of connective tissues), and atherosclerosis.

Suitably the proliferative disease is selected from bone cancer, bowel cancer, brain cancer, breast cancer, CNS cancer, cervical cancer, colon cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma, ovarian cancer, pancreatic cancer, prostate cancer, renal cancer, skin cancer, stomach cancer and thyroid cancer.

More suitably the proliferative disease is selected from bone cancer, breast cancer, colon cancer, liver cancer, lung cancer and stomach cancer.

In some embodiments, the proliferative disease is selected from adenosquamous carcinoma, colorectal carcinoma, Dukes' type B colorectal adenocarcinoma, gastric adenocarcinoma, hepatoblastoma, non-small cell lung cancer, osteosarcoma and triple negative breast cancer.

A skilled person is readily able to determine whether or not a candidate compound treats a proliferative condition for any particular cell type.

Suitably subjects are human, livestock animals and companion animals.

Most suitably the subjects are human.

Administration & Dose

Compounds of formula (I) may be administered alone or in combination with one or another or with one or more pharmacologically active compounds which are different from the compounds of formula (I). Suitably, the one or more pharmacologically active compounds are selected from compounds suitable for treating anti-proliferative diseases and salts and solvates thereof. Suitably, the one or more pharmacologically active compounds are selected from compounds suitable for treating anti-proliferative diseases and salts and solvates thereof, inhibitors of glutamate cysteine ligase and mixtures thereof. The one or more pharmacologically active compounds, more suitably, comprises one or two pharmacologically active compound and salts and solvates thereof.

Suitably, the 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof for use in the treatment of a proliferative disease, is administered, either simultaneously or sequentially, with one or more pharmacologically active compounds and salts and solvates thereof.

Suitably, the one or more pharmacologically active compounds are selected from compounds suitable for treating anti-proliferative diseases and salts and solvates thereof. More suitably, the one more pharmacologically active compounds and salts and solvates thereof suitable for treating anti-proliferative diseases are selected from alkylating agents, platinum compounds, DNA altering compounds, microtubule modifiers, antimetabolites, anticancer antibodies, small molecule kinase inhibitors, drug conjugates, miscellaneous antitumor agents and mixtures thereof.

Suitably, the alkylating agents may be selected from altretamine, bendamustine, busulfan, carmustine, chlorambucil, chlormethine, cyclophosphamide, dacarbazine, ifosfamide, improsulfan tosilate, lomustine, melphalan, mitobronitol, mitolactol, nimustine, ranimustine, steptozoin, temozolomide, thiotepa, treosulfan, mechloretamine, carboquone; apaziquone, fotemustine, glufosfamide, palifosfamide, pipobroman, trofosfamide, uramustine, and mixtures and salts and solvates thereof.

Suitably, the platinum compounds may be selected from carboplatin, cisplatin, eptaplatin, miriplatine hydrate, oxaliplatin, lobaplatin, nedaplatin, picoplatin, satraplatin, and mixtures and salts and solvates thereof.

Suitably, the DNA altering agents may be selected from amrubicin, bisantrene, decitabine, mitoxantrone, procarbazine, trabectedin, clofarabine, amsacrin, brostallicin, pixantrone, laromustine, and mixtures and salts and solvates thereof.

Suitably, the microtubule modifiers may be selected from albendazole, cabazitaxel, ciclobendazole, colchicine, docetaxel, eribulin, flubendazole, fosbretabulin, griseofulvin, ixabepilone, mebendazole, paclitaxel, podophyllotoxin, quinfamide, secnidazole, triclabendazole, vinblastine, vincristine, vinorelbine, vindesine, vinflunine, tesetaxel, and mixtures and salts and solvates thereof.

Suitably, the antimetabolites may be selected from asparaginase, azacitidine, calcium levofolinate, capecitabine, cladribine, claribine, cytarabine, enocitabine, floxuridine, fludarabine, fluorouracil, hydroxyurea, gemcitabine, mercaptopurine, methotrexate, nelarabine, pemetrexed, pralatrexate, azathioprine, thioguanine, carmofur, doxifluridine, elacytarabine, raltitrexed, sapacitabine, tegafur, trimetrexate, and mixtures and salts and solvates thereof.

Suitably, the anticancer antibiotics may be selected from bleomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, levamisole, miltefosine, mitomycin C, romidepsin, streptozocin, valrubicin, zinostatin, zorubicin, daunurobicin, plicamycin, aclarubicin, peplomycin, pirarubicin, and mixtures and salts and solvates thereof.

Suitably, the small molecule kinase inhibitors may be selected from crizotinib, dasatinib, erlotinib, imatinib, lapatinib, nilotinib, pazopanib, regorafenib, ruxolitinib, sorafenib, sunitinib, vandetanib, vemurafenib, bosutinib, gefitinib, axitinib; afatinib, alisertib, dabrafenib, dacomitinib, dinaciclib, dovitinib, enzastaurin, nintedanib, lenvatinib, linifanib, linsitinib, masitinib, midostaurin, motesanib, neratinib, orantinib, perifosine, ponatinib, radotinib, rigosertib, tipifarnib, tivantinib, tivozanib, trametinib, pimasertib, brivanib alaninate, cediranib, apatinib, cabozantinib S-malate, ibrutinib, icotinib, buparlisib, cipatinib, cobimetinib, idelalisib, fedratinib, and mixtures and salts and solvates thereof.

Suitably, the drug conjugates may be selected from denileukin diftitox, ibritumomab tiuxetan, iobenguane I123, prednimustine, trastuzumab emtansine, estramustine, gemtuzumab, ozogamicin, aflibercept; cintredekin besudotox, edotreotide, inotuzumab ozogamicin, naptumomab estafenatox, oportuzumab monatox, technetium (99mTc) arcitumomab, vintafolide, and mixtures and salts and solvates thereof.

Suitably, the miscellaneous antitumor agents may be selected from alitretinoin, bexarotene, bortezomib, everolimus, ibandronic acid, imiquimod, lenalidomide, lentinan, metirosine, mifamurtide, pamidronic acid, pegaspargase, pentostatin, sipuleucel, sizofiran, tamibarotene, temsirolimus, thalidomide, tretinoin, vismodegib, zoledronic acid, vorinostat; celecoxib, cilengitide, entinostat, etanidazole, ganetespib, idronoxil, iniparib, ixazomib, lonidamine, nimorazole, panobinostat, peretinoin, plitidepsin, pomalidomide, procodazol, ridaforolimus, tasquinimod, telotristat, thymalfasin, tirapazamine, tosedostat, trabedersen, ubenimex, valspodar, gendicine, picibanil, reolysin, retaspimycin hydrochloride, trebananib, virulizin, carfilzomib, endostatin, immucothel, belinostat, and mixtures and salts and solvates thereof.

The above pharmacologically active compounds and salts and solvates thereof, are available from a variety of sources, such as, AstraZeneca, Bayer, Bristol-Meyers Squibb, Genetech, GlaxoSmithKline, Merck, Novartis, Pfizer and Roche.

Compounds of the invention may suitably be combined with various components to produce compositions of the invention. Suitably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. Useful pharmaceutical compositions and methods for their preparation may be found in standard pharmaceutical texts. See, for example, *Handbook for Pharmaceutical Additives*, 3rd Edition (eds. M. Ash and I. Ash), 2007 (Synapse Information Resources, Inc., Endicott, New York, USA) and *Remington: The Science and Practice of Pharmacy*, 21st Edition (ed. D. B. Troy) 2006 (Lippincott, Williams and Wilkins, Philadelphia, USA) which are incorporated herein by reference.

The compounds of the invention may be administered by any suitable route. Suitably the compounds of the invention will normally be administered orally or by any parenteral route, in the form of pharmaceutical preparations comprising the active

ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in a pharmaceutically acceptable dosage form.

The compounds of the invention, their pharmaceutically acceptable salts, and pharmaceutically acceptable solvates of either entity can be administered alone but will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the compounds of the invention or salts or solvates thereof can be administered orally, buccally or sublingually in the form of tablets, capsules (including soft gel capsules), ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed-, modified-, sustained-, controlled-release or pulsatile delivery applications. The compounds of the invention may also be administered via fast dispersing or fast dissolving dosages forms.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethyl cellulose (HPMC), hydroxypropylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

Modified release and pulsatile release dosage forms may contain excipients such as those detailed for immediate release dosage forms together with additional excipients that act as release rate modifiers, these being coated on and/or included in the body of the device. Release rate modifiers include, but are not exclusively limited to, hydroxypropylmethyl cellulose, methyl cellulose, sodium carboxymethylcellulose, ethyl

cellulose, cellulose acetate, polyethylene oxide, Xanthan gum, Carbomer, ammonio methacrylate copolymer, hydrogenated castor oil, carnauba wax, paraffin wax, cellulose acetate phthalate, hydroxypropylmethyl cellulose phthalate, methacrylic acid copolymer and mixtures thereof. Modified release and pulsatile release dosage forms may contain one or a combination of release rate modifying excipients. Release rate modifying excipients maybe present both within the dosage form i.e. within the matrix, and/or on the dosage form i.e. upon the surface or coating.

Fast dispersing or dissolving dosage formulations (FDDFs) may contain the following ingredients: aspartame, acesulfame potassium, citric acid, croscarmellose sodium, crospovidone, diascorbic acid, ethyl acrylate, ethyl cellulose, gelatin, hydroxypropylmethyl cellulose, magnesium stearate, mannitol, methyl methacrylate, mint flavouring, polyethylene glycol, fumed silica, silicon dioxide, sodium starch glycolate, sodium stearyl fumarate, sorbitol, xylitol.

A variety of advanced drug delivery systems have been developed and may be used with the compounds and compositions. For example, the compounds and compositions may be formulated as liposomes, micelles, nanocarriers and combinations thereof optionally coated with polyethylene glycol, ligand(s) and/or antibodies. Such formulations can reduce toxicity and increase the stability/half-life of the drug.

Liposomes are a bilayered phospholipid vesicles typically from approximately 50 to 1,000 nm in diameter. Liposomes can carry a variety of water soluble and water insoluble drugs loaded in an inner aqueous compartment or into a phospholipid bilayer. Liposomes are biologically inert and completely biocompatible; they cause practically no toxic or antigenic reactions. The ability of liposomes as drug carriers have been demonstrated in numerous laboratory tests and clinical trials, e.g., Torchilin, *Nat. Rev. Drug Discov.* 4, 145-160 (2005)

Micelles are self-assembling spherical colloidal nanoparticles formed by amphiphilic molecules; they can also be described as aggregate surfactant molecules disbursed in a liquid colloid. Hydrophobic fragments of amphiphilic molecules form the core of a micelle while their hydrophilic heads form a micelle corona. The core of the micelle is capable of encapsulating drugs protecting them. Micelles are generally from approximately 5 to 50 nm in diameter. Micelles may be formed by any of commonly known surfactants, such as sodium dodecylsulfate or phospholipids, but the performance of such surfactants as drug delivery systems is low compared to micelles

composed of specially designed block copolymers, as described in Kataoka et al., *J. Control Release* 64, 143-153 (2000). Such polymer micelles dissociate much more slowly than unmodified surfactant micelles, retain the loaded drugs for a longer period of time and accumulate the drug at the target site more efficiently.

Nanocarriers, also referred to as 'nanoparticles', have been described by Gu et al., 2011, *Chem. Soc. Rev.* 40:3638-3655 and by Gunaseelan et al., 2010, *Adv. Drug Deliv. Rev.* 62:518-531. Nanoparticles can be constructed with a variety of nanomaterials, such as those described in Al-Jamal et al., 2010, *FASEB J.* 24:4354-4365; Adeli et al., 2011, *Nanomedicine* 7:806-817; Bulut et al., 2011, *Biomacromolecules* 12:3007-3014.

Hybrid nanocarrier systems, which consist of components of two or more particulate delivery systems, may be used. Examples of hybrid narocarrier systems include those described in Pittella et al., 2011, *Biomaterials* 32:3106-3114; polymeric micelle nanocarrier, such as those described in Chen et al., 2011, *Biomacromolecules* 12:3601-3611; and liposomal nanocarriers, such as those described in Kang et al., 2011, *J. Drug Target* 19:497-505.

Liposomes and micelles can be stabilized by enhancing the outermost hydrophobic shell with water soluble polymers, such as polyethyleneglycol (PEG). The presence of hydrophilic polymers on the hydrophobic surface of these carrier particles, results in a decrease in both the rate and extent of uptake of carrier particles by mononuclear phagocytes. Several preparations based on long circulating liposomes are commercially available, for example, Doxil.RTM., a doxorubicin containing polyethyleneglycolated (PEGylated) liposomes, Sharp et al., *Drugs* 62 2089-2126 (2002). Doxil is manufactured by Ortho Biotech Products, LP of Bridgewater, N.J., USA.

These carrier particles have been modified with various ligands using advance conjugation procedures to achieve more specific targeting to the sites of interest. For example, antibodies and small peptides have been attached to the water exposed tips of polyethyleneglycol chains, Blume, et al. *Biomembranes* 1149, 180-184 (1993). Antibodies and small peptides have also been conjugated via reactive p-nitrophenylcarbonyl or maleimide terminated PEG-phosphatidylethanolamine, Moreira, *Pharm. Res.* 19, 265-269 (2002) and Xiong, et al., *J. Pharm. Sci.* 94, 1782-1793 (2005).

The compounds of the invention can also be administered parenterally, for example, intravenously, intra-arterially, or they may be administered by infusion techniques. For such parenteral administration they are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

Suitably formulation of the invention is optimised for the route of administration e.g. oral, intravenously, etc.

Administration may be in one dose, continuously or intermittently (e.g. in divided doses at appropriate intervals) during the course of treatment. Methods of determining the most effective means and dosage are well known to a skilled person and will vary with the formulation used for therapy, the purpose of the therapy, the target cell(s) being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and the dose regimen being selected by the treating physician, veterinarian, or clinician.

Depending upon the disorder and patient to be treated, as well as the route of administration, the compositions may be administered at varying doses. For example, a typical dosage for an adult human may be 100 ng to 25 mg (suitably about 1 micro g to about 10 mg) per kg body weight of the subject per day.

Suitably guidance may be taken from studies in test animals when estimating an initial dose for human subjects. For example when a particular dose is identified for mice, suitably an initial test dose for humans may be approx. 0.5x to 2x the mg/Kg value given to mice.

Synthesis

A wide variety of compounds of Formula (I) are commercially available, for example 5-bromo-2-methylsulfonyl-4-pyrimidinecarboxylic acid, and may be purchased from companies such as Enamine, Vitas-M laboratory (Moscow, Russian Federation), and Key Organics (Cornwall, UK). Derivatives of such commercially available compounds may be prepared by carrying out functional group interconversions or making substitutions and carrying out common reactions as are known in the art. The

synthesis of various 2-sulfonylpyrimidine compounds is described (56, 57, 58, 59) and in US4,711,959, US2002198206, US2003135047, WO2004018428, US2007056221, US2009069180, WO2011034907, US2015094205 and US2015183784. In several of these documents the 2-sulfonyl-pyrimidine compounds are synthesised as intermediates and in many cases the 2-sulfonylpyrimidine compounds are formed by oxidation of the corresponding thio ether compound, using reagents such as hydrogen peroxide, 3-chloroperbenzoic acid or oxone. Common techniques and reactions to prepare further derivatives, including oxidations, reductions, and so on, separation techniques (extraction, evaporation, precipitation, chromatography, filtration, trituration, crystallization, and the like), and analytical procedures, are known to persons of ordinary skill in the art of organic chemistry. The details of such reactions and techniques can be found in a number of treatises, including Richard Larock, Comprehensive Organic Transformations, A Guide to Functional Group Preparations, 2nd Ed (2010), and the multi-volume series edited by Michael B. Smith and others, Compendium of Organic Synthetic Methods (1974 et seq.). Starting materials and reagents may be obtained from commercial sources or may be prepared using literature methods. In some instances, reaction intermediates may be used in subsequent steps without isolation or purification (i.e., in situ).

Certain compounds can be prepared using protecting groups, which prevent undesirable chemical reaction at otherwise reactive sites. Protecting groups may also be used to enhance solubility or otherwise modify physical properties of a compound. For a discussion of protecting group strategies, a description of materials and methods for installing and removing protecting groups, and a compilation of useful protecting groups for common functional groups, including amines, carboxylic acids, alcohols, ketones, aldehydes, and so on, see T. W. Greene and P. G. Wuts, Protecting Groups in Organic Chemistry, 4th Edition, (2006) and P. Kocienski, Protective Groups, 3rd Edition (2005).

Generally, chemical transformations may be carried out using substantially stoichiometric amounts of reactants, though certain reactions may benefit from using an excess of one or more of the reactants. Additionally, many of the suitable reactions may be carried out at about room temperature (RT) and ambient pressure, but depending on reaction kinetics, yields, and so on, some reactions may be run at elevated pressures or employ higher temperatures (e.g., reflux conditions) or lower temperatures (e.g., -78°C. to 0°C.). Any reference in the disclosure to a stoichiometric

range, a temperature range, a pH range, etc., whether or not expressly using the word "range," also includes the indicated endpoints.

Many of the chemical transformations may also employ one or more compatible solvents, which may influence the reaction rate and yield. Depending on the nature of the reactants, the one or more solvents may be polar protic solvents (including water), polar aprotic solvents, non-polar solvents, or some combination. Representative solvents include saturated aliphatic hydrocarbons (e.g., n-pentane, n-hexane, n-heptane, n-octane); aromatic hydrocarbons (e.g., benzene, toluene, xylenes); halogenated hydrocarbons (e.g., methylene chloride, chloroform, carbon tetrachloride); aliphatic alcohols (e.g., methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol, 2-methyl-propan-1-ol, butan-2-ol); ethers (e.g., diethyl ether, di-isopropyl ether, dibutyl ether, 1,2-dimethoxy-ethane, tetrahydrofuran, 1,4-dioxane); ketones (e.g., acetone, methyl ethyl ketone); esters (methyl acetate, ethyl acetate); nitrogen-containing solvents (e.g., formamide, N,N-dimethylformamide, acetonitrile, N-methyl-pyrrolidone, pyridine, quinoline, nitrobenzene); sulfur-containing solvents (e.g., carbon disulfide, dimethyl sulfoxide, tetrahydro-thiophene-1,1,-dioxide); and phosphorus-containing solvents (e.g., hexamethylphosphoric triamide).

Other Forms

Unless otherwise specified, included in the above are the well known ionic, salt, solvate, and protected forms of these substituents. For example, a reference to carboxylic acid (-COOH) also includes the anionic (carboxylate) form ($-COO^-$), a salt or solvate thereof, as well as conventional protected forms. Similarly, a reference to an amino group includes the protonated form ($-N^+HR^1R^2$), a salt or solvate of the amino group, for example, a hydrochloride salt, as well as conventional protected forms of an amino group. Similarly, a reference to a hydroxyl group also includes the anionic form ($-O^-$), a salt or solvate thereof, as well as conventional protected forms.

Isomers, Salts and Solvates

Certain compounds may exist in one or more particular geometric, optical, enantiomeric, diasteriomeric, epimeric, atropic, stereoisomeric, tautomeric, conformational, or anomeric forms, including but not limited to, cis- and trans-forms; E- and Z-forms; c-, t-, and r- forms; endo- and exo-forms; R-, S-, and meso-forms; D- and L-forms; d- and l- forms; (+) and (-) forms; keto-, enol-, and enolate-forms; syn- and anti-forms; synclinal- and anticlinal-forms; alpha- and beta-forms; axial and equatorial forms; boat-, chair-, twist-, envelope-, and halfchair-forms; and

combinations thereof, hereinafter collectively referred to as "isomers" (or "isomeric forms").

Note that, except as discussed below for tautomeric forms, specifically excluded from the term "isomers", as used herein, are structural (or constitutional) isomers (i.e. isomers which differ in the connections between atoms rather than merely by the position of atoms in space). For example, a reference to a methoxy group, $-OCH_3$, is not to be construed as a reference to its structural isomer, a hydroxymethyl group, $-CH_2OH$.

A reference to a class of structures may well include structurally isomeric forms falling within that class (e.g. C_{1-6} alkyl includes n-propyl and iso-propyl; butyl includes n-, iso-, sec-, and tert-butyl; methoxyphenyl includes ortho-, meta-, and para-methoxyphenyl).

The above exclusion does not apply to tautomeric forms, for example, keto-, enol-, and enolate-forms, as in, for example, the following tautomeric pairs: keto/enol, imine/enamine, amide/imino alcohol, amidine/amidine, nitroso/oxime, thioketone/enethiol, N-nitroso/hyroxyazo, and nitro/aci-nitro.

Note that specifically included in the term "isomer" are compounds with one or more isotopic substitutions. For example, H may be in any isotopic form, including ^{1}H, ^{2}H (D), and ^{3}H (T); C may be in any isotopic form, including ^{12}C, ^{13}C, and ^{14}C; O may be in any isotopic form, including ^{16}O and ^{18}O; and the like.

Unless otherwise specified, a reference to a particular compound includes all such isomeric forms, including (wholly or partially) racemic and other mixtures thereof.

Methods for the preparation (e.g. asymmetric synthesis) and separation (e.g. fractional crystallisation and chromatographic means) of such isomeric forms are either known in the art or are readily obtained by adapting the methods taught herein, or known methods, in a known manner.

Unless otherwise specified, a reference to a particular compound also includes ionic, salt, solvate, and protected forms of thereof, for example, as discussed below.

Compounds of Formula (I), which include compounds specifically named above, may form pharmaceutically acceptable complexes, salts, solvates and hydrates. These salts include nontoxic acid addition salts (including di-acids) and base salts.

If the compound is cationic, or has a functional group which may be cationic (e.g. $-NH_2$ may be $-NH_3^+$), then an acid addition salt may be formed with a suitable anion. Examples of suitable inorganic anions include, but are not limited to, those derived from the following inorganic acids hydrochloric acid, nitric acid, nitrous acid, phosphoric acid, sulfuric acid, sulphurous acid, hydrobromic acid, hydroiodic acid, hydrofluoric acid, phosphoric acid and phosphorous acids. Examples of suitable organic anions include, but are not limited to, those derived from the following organic acids: 2-acetyoxybenzoic, acetic, ascorbic, aspartic, benzoic, camphorsulfonic, cinnamic, citric, edetic, ethanedisulfonic, ethanesulfonic, fumaric, glucheptonic, gluconic, glutamic, glycolic, hydroxymaleic, hydroxynaphthalene carboxylic, isethionic, lactic, lactobionic, lauric, maleic, malic, methanesulfonic, mucic, oleic, oxalic, palmitic, pamoic, pantothenic, phenylacetic, phenylsulfonic, propionic, pyruvic, salicylic, stearic, succinic, sulfanilic, tartaric, toluenesulfonic, and valeric. Examples of suitable polymeric organic anions include, but are not limited to, those derived from the following polymeric acids: tannic acid, carboxymethyl cellulose. Such salts include acetate, adipate, aspartate, benzoate, besylate, bicarbonate, carbonate, bisulfate, sulfate, borate, camsylate, citrate, cyclamate, edisylate, esylate, formate, fumarate, gluceptate, gluconate, glucuronate, hexafluorophosphate, hibenzate, hydrochloride/chloride, hydrobromide/bromide, hydroiodide/iodide, isethionate, lactate, malate, maleate, malonate, mesylate, methylsulfonate, naphthylate, 2-napsylate, nicotinate, nitrate, orotate, oxalate, palmitate, pamoate, phosphate, hydrogen phosphate, dihydrogen phosphate, pyroglutamate, saccharate, stearate, succinate, tannate, tartrate, tosylate, trifluoroacetate and xinofoate salts.

For example, if the compound is anionic, or has a functional group which may be anionic (e.g. -COOH may be $-COO^-$), then a base salt may be formed with a suitable cation. Examples of suitable inorganic cations include, but are not limited to, metal cations, such as an alkali or alkaline earth metal cation, ammonium and substituted ammonium cations, as well as amines. Examples of suitable metal cations include sodium (Na^+) potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), zinc (Zn^{2+}), and aluminum (Al^{3+}). Examples of suitable organic cations include, but are not limited to, ammonium ion (i.e. $NH4^+$) and substituted ammonium ions (e.g. NH_3R^+, $NH_2R_2^+$, NHR_3^+, NR_4^+). Examples of some suitable substituted ammonium ions are those

derived from: ethylamine, diethylamine, dicyclohexylamine, triethylamine, butylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine, benzylamine, phenylbenzylamine, choline, meglumine, and tromethamine, as well as amino acids, such as lysine and arginine. An example of a common quaternary ammonium ion is $N(CH_3)_4^+$. Examples of suitable amines include arginine, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethylamine, diethanolamine, dicyclohexylamine, ethylenediamine, glycine, lysine, N-methylglucamine, olamine, 2-amino-2-hydroxymethyl-propane-1,3-diol, and procaine. For a discussion of useful acid addition and base salts, see S. M. Berge et al., *J. Pharm. Sci.* (1977) 66:1-19; see also Stahl and Wermuth, *Handbook of Pharmaceutical Salts: Properties, Selection, and Use* (2011)

Pharmaceutically acceptable salts may be prepared using various methods. For example, one may react a compound of Formula (I) with an appropriate acid or base to give the desired salt. One may also react a precursor of the compound of Formula (I) with an acid or base to remove an acid- or base-labile protecting group or to open a lactone or lactam group of the precursor. Additionally, one may convert a salt of the compound of Formula (I) to another salt through treatment with an appropriate acid or base or through contact with an ion exchange resin. Following reaction, one may then isolate the salt by filtration if it precipitates from solution, or by evaporation to recover the salt. The degree of ionization of the salt may vary from completely ionized to almost non-ionized.

It may be convenient or desirable to prepare, purify, and/or handle a corresponding solvate of the active compound. The term "solvate" describes a molecular complex comprising the compound and one or more pharmaceutically acceptable solvent molecules (e.g., EtOH). The term "hydrate" is a solvate in which the solvent is water. Pharmaceutically acceptable solvates include those in which the solvent may be isotopically substituted (e.g., D_2O, acetone-d6, DMSO-d6).

A currently accepted classification system for solvates and hydrates of organic compounds is one that distinguishes between isolated site, channel, and metal-ion coordinated solvates and hydrates. See, e.g., K. R. Morris (H. G. Brittain ed.) Polymorphism in Pharmaceutical Solids (1995). Isolated site solvates and hydrates are ones in which the solvent (e.g., water) molecules are isolated from direct contact with each other by intervening molecules of the organic compound. In channel solvates, the

solvent molecules lie in lattice channels where they are next to other solvent molecules. In metal-ion coordinated solvates, the solvent molecules are bonded to the metal ion.

When the solvent or water is tightly bound, the complex will have a well-defined stoichiometry independent of humidity. When, however, the solvent or water is weakly bound, as in channel solvates and in hygroscopic compounds, the water or solvent content will depend on humidity and drying conditions. In such cases, non-stoichiometry will typically be observed.

These compounds may be isolated in solid form, for example, by lyophilisation.

Further particular and preferred aspects are set out in the accompanying independent and dependent claims. Features of the dependent claims may be combined with features of the independent claims as appropriate, and in combinations other than those explicitly set out in the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present invention will now be described further, with reference to the accompanying drawings, in which:

Figure 1 (A) shows a melting curve of stabilized, full-length p53 (T-p53) recorded via differential scanning fluorimetry in absence (blue) or in presence of 1 mM PK11000 (magenta). Figure 1 (B) shows the mapping of PK11000 induced peak shifts in the ^{15}N-^{1}H-HSQC NMR spectrum onto the structure of the p53-Y220C core domain. Large peak shifts are highlighted in red, medium shifts in orange and small shifts in yellow. Figure 1 (C) shows the inhibition of T-p53C-Y220C aggregation by PK11000.

Figure 2 shows how PK11000 alkylates cysteines of the p53 core domain. Figure 2 (A) shows a SNAr reaction mechanism for PK11000 cysteine alkylation. Figure 2 (B) shows an ESI (ES+) mass spectra of 50 μM T-p53C Y220C incubated for 4 hours at 20 °C with no compound (black) or 250, 500, 1000, and 5000 μM PK11000 (red).

Figure 3 shows ESI (ES+) mass spectra of different cysteine to serine p53 core domain mutants after incubation without (black) and with PK11000 (red). Figure 3 (A) relating to T-p53C C124S/C182S/C277S and (D) relating to T-p53C C182S/C277S mutants showed no cysteine modification by PK11000, whereas (B) relating to T-p53C

C124S/C182S and (C) relating to T-p53C C124S/C277S showed one cysteine modification by PK11000.

Figure 4 shows the structural effect of Cys182 modification by PK11000. Superposition of the structure of the p53 cancer mutant Y220C with (gray) and without (green) PK11000 shows that Cys182 on the surface of the L2 loop is modified by the alkylating agent, with the covalent modification pointing towards the solvent. Alkylation fixes the Cys182 side chain and the Cys182/Ser183 backbone in a defined conformation, but there is little interaction between the modification and the rest of the protein. The figure was generated using PyMOL (www.pymol.org).

Figure 5 shows ^{1}H-NMR kinetic measurement of PK11000-glutathione adduct formation. Adduct concentrations were calculated by peak integration of an aromatic product peak at 8.52 ppm.

Figure 6 shows the effects of diverse 2-sulfonylpyrimdines on p53 stabilization. Figure 6 (A) shows a library of diverse 2-sulfonylpyrmidines and structurally related compounds. Figure 6 (B) and (C) show the time-dependent stabilization (DSF ΔT_m) of T-p53C-Y220C after 15, 30, 60, and 120 minutes incubation at room temperature with stabilizing or destabilizing/non-reactive 2-sulfonylpyrmidine compounds, respectively.

Figure 7 shows the biological effects of PK11007 on diverse cancer cell lines. Figure 7 (A) shows concentration-dependent viability reduction of NUGC-4 (p53 wt), HUH-6 (p53 wt), SJSA-1 (p53 wt), SW480 (p53 R273H/P309S), NUGC-3 (p53 Y220C), HUH-7 (p53 Y220C), and MKN1 (p53 V143A) cells after treatment with PK11007 for 24 hours. The mutant p53 cells HUH-7 and NUGC-3 were significantly more sensitive to PK11007 treatment as indicated by strong viability reduction at low compound concentrations. Figure 7 (B)shows incubation of the isogenic H1299 (p53 -/-), H1299 (p53 H175) and HCT116, HCT116 p53 -/- cancer cells with PK11007 yielded a comparable viability reduction after 24 hours. Figure 7 (C) shows inhibition of cellular glutathione synthesis by buthionine sulfoximine (BSO) strongly potentiated cell viability reduction by PK11007 in HUH-7, NUGC-3, and MKN1 mutant p53 cancer cells. Figure 7 (D) shows the determination of relative intracellular ROS levels via CellROX Deep Red fluorescence after incubating four cancer cell lines with 30 or 60 μM PK11007 for 2 hours. PK11007 lead to an increase of ROS in all tested cell lines, however, at high doses the increase of relative ROS levels was significantly higher in HUH-7, NUGC-3

and MKN1 cells. Median fluorescence levels were determined in triplicate with error bars depicting the SE.

Figure 8 shows a ^{15}N-^{1}H-HSQC NMR spectrum of the p53 Y220C core domain (red) with 1000 μM (blue), 436 μM (yellow), and 218 μM (green) PK11000 at 298K.

Figure 9 shows that alkylation of full-length p53 with 2-sulfonylpyrimdines does not compromise its DNA binding capability. Stabilized full-length p53 was incubated with 1 mM PK11000, PK11007, PK11010, and 5% DMSO and was then titrated into a cuvette containing carboxyfluoresceine labelled Gadd45a DNA and fluorescence polarization data was recorded.

Figure 10 shows viability reduction of APR-246 in NUGC-3 (p53 wt), HUH-6 (p53 wt), NUGC-4 (p53 Y220C), and HUH-7 (p53 Y220C) cancer cells after treatment for 24 hours.

Figure 11 shows cell viability time course for HUH-6 (p53 wt) and HUH-7 (p53 Y220C) [Figure 11 A] and MKN1 [Figure 11B] cancer cells after incubation with 30 or 60 μM PK11007 (10 and 30 μM for MKN1). Cell viability was measured in quadruplicates and normalized with the values of blank (viability = 1) and no cell (viability = 0) Q:3 controls. Data are shown as mean ± SEM

Figure 12 shows that N-acetylcysteine (NAC) prevents PK11007 mediated ROS formation. Figure 12 (A) shows the determination of intracellular ROS levels via CellROX Deep Red fluorescence after incubating HUH-6 and HUH-7 cancer cells with 5 mM NAC, 400 μM tert-buyl hydroperoxide (TBHP), or a combination of 30 μM PK11007 and 5mM NAC for 1.25 hours. NAC not only decrease basal ROS levels, but also completely prevents PK11007 from increasing ROS levels. Figure 12 (B) shows the relative intracellular ROS levels via CellROX Deep Red fluorescence after incubating four cancer cell lines with 30 or 60 μM PK11007 for 2 hours. PK11007 lead to an increase of ROS in all tested cell lines, however, at high doses the relative ROS level was significantly higher in HUH-7 and NUGC-3 cells, while the ROS levels in MKN1 cells remained on the level of the p53 WT cell lines.

Figure 13 shows that PK11007 can induce cell death via caspase-independent pathways. HUH-6 and HUH-7 cells were treated for 6 hours with PK11007, APR-246, and Nutlin-3. Figure 13 (A) shows that PK11007 and APR-246 treatment did not significantly

increase caspase 3 and caspase 7 activities, only Nutlin-3 yielded a significant caspase induction in HUH-6 cells. Figure 13 (B) shows that membrane permeability was significantly increased for high PK11007 concentrations or in combination with BSO in HUH-7 cells, whereas HUH-6 cells were not affected. APR-246 and Nutlin-3 did not significantly induce cytotoxicity. Figure 13 (C) shows that PK11007 slightly increases caspase 3/7 activity in SW480 (at 15 μM), SJSA-1 (at 30 μM), and HCT116 (at 60 μM) cancer cells.

Figure 14 shows a western blot that demonstrates the effects of PK11007 on p53 and p21 protein levels in HUH-6 (p53-WT) and HUH-7 (p53-Y220C) cells.

Figure 15A and B show cell viability data for various PK11xxx compounds in diverse cancer cell lines and one normal fibroblast cell line (WI-38, non-cancer).

Figure 16 shows the effects of compound PK11007 on cell viability in a panel of breast cancer cell lines.

Figure 17 shows the effects of compound PK11000 on proliferation in a panel of breast cancer cell lines.

Figure 18 shows the effects of compound PK11007 on proliferation in a panel of breast cancer cell lines.

Figure 19 shows the effects of compound PK11010 on proliferation in a panel of breast cancer cell lines.

Figure 20 shows the relationship between the response to PK11000/PK11007/PK11010 and molecular subtype.

Figure 21 shows the relationship between the response to PK11007 and p53 protein level.

Figure 22 shows the relationship between PK11007 compounds and PRIMA-1^{MET}.

Figure 23 shows the effects of p53 knockdown on cell line response.

Figure 24 shows the effects of p53 knockdown on PK11007 response.

Figure 25 shows the cell viability of HUH-6, HUH-7, and MKN1 after p53 knockdown via siRNA. Cells were treated with PK11007 for 24 h (48 h for 15 μM PK11007 MKN1 sample).

Figure 26 shows western blots of NUGC-4, NUGC-3, MKN1, HUH-6, and HUH-7 cancer cells after 3 h (6 h for MKN1) treatment with PK11007.

Figure 27 shows quantification of relative mRNA levels of p53 target genes via real-time PCR.

Figure 28 shows western blots of protein levels of UPR key markers in MKN1, HUH-6, and HUH-7 cells after PK11007 treatment for 3 h (6 h for MKN1).

EXAMPLES

MATERIALS & METHODS

Materials

The in-house fragment library used for the DSF screening assay was purchased from Enamine (Kiev, Ukraine) in 96-well plate format at 20mM compound concentrations in DMSO. Plates were stored at -20 °C. Derivatives of PK11000 were purchased from Enamine, Vitas-M laboratory (Moscow, Russian Federation), and Key Organics (Cornwall, UK). APR-246 (PRIMA-1^{MET}) and buthionine sulfoximine (BSO) were purchased from Santa Cruz Biotechnology. DMEM High Glucose GlutaMAX and RPMI 1640 Medium GlutaMAX were obtained from Life Technologies Ltd.

Protein Expression and Purification.

Plasmids for expression of the cysteine mutants C124/277S, C124/182S, and C182/277S of the p53 core domain were generated with the Quikchange II site-directed mutagenesis kit (Agilent Technologies) according to the instructions of the manufacturer. A pET24a vector with the stabilized p53 core domain was used as template (15). The stabilized DNA-binding domain of the p53 mutant Y220C (T-p53C-Y220C) and T-p53C cysteine mutants were expressed and purified as described (10). Escherichia coli N-acetylneuraminate lyase was produced as described previously (44).

Differential Scanning Fluorimetry

Melting temperatures of p53 variants were determined by DSF measurements using SYPRO Orange (Invitrogen) as the fluorescent probe as described (10). A final protein

concentration of 8 μM in standard phosphate buffer (25 mM potassium phosphate pH 7.2, 150 mM NaCl, 1 mM TCEP, 5% (v/v) DMSO) was used for the DSF measurements. ΔT_m DSF values were calculated by subtracting the average T_m of the control samples from the average T_m of the respective compound samples. All samples were measured in triplicate.

HSQC-NMR

$^{1}H/^{15}N$-HSQC spectra of uniformly ^{15}N-labeled T-p53C-Y220C (75 μM) compounds were recorded and analysed as described (10). Briefly, the spectra were acquired at 293K on a Bruker Avance-800 spectrometer using a 5-mm inverse cryogenic probe. Compound samples were mixed with protein immediately before the NMR measurement. Spectra analysis was performed using Sparky 3.11430 and Bruker Topspin 2.0 software.

Aggregation Kinetics

Aggregation kinetics of the p53 Y220C core domain (94-312) was measured as described (10). Briefly, light scattering was recorded at 37 °C at 500 nm as excitation and emission wavelengths using a Horiba FluoroMax-3 spectrophotometer. Experiments were performed in standard phosphate buffer (25 mM KPi pH7.2, 150 mM NaCl, 1 mM TCEP and 5% DMSO) with 3 μM protein.

X-ray crystallography

Crystals of T-p53C-Y220C were grown as described (45). They were soaked for 4 h in a solution of 30 mM PK11000 in cyro buffer (19% polyethylene glycol 4000, 20% glycerol, 10 mM sodium phosphate, pH 7.2, 100 mM Hepes, pH 7.2, 150 mM NaCl), and flash frozen in liquid nitrogen. An X-ray data set was collected at 100 K at beamline I03 of the Diamond Light Source. The data set was integrated using XDS (46) and scaled using SCALA (47) within the CCP4 suite of programs (48). The structure was determined by rigid body refinement in PHENIX (49) using PDB entry 2J1X as a starting model, and subsequently refined with iterative cycles of manual model building in COOT (50) and refinement with REFMAC5 (51). Data collection and refinement statistics are given in Table SX.

Mass Spectrometry

To check for alkylation of T-p53C-Y220C (94-312) and T-p53, DMSO stocks of compounds were added to a reaction buffer containing 50 μM protein, 25mM potassium phosphate pH 7.2, 150 mM sodium chloride, 1mM TCEP, yielding a final

concentration of 5% DMSO. The samples were incubated for 4 hours at 20°C on a rotating shaker. The protein sample was then diluted to 5 μM with 100 mM ammonium acetate buffer and desalted using Millipore (Bedford, MA) C4 zip-tips. The mass of the proteins was determined by electrospray mass spectrometry with a Waters (Micromass) LCT TOF mass spectrometer in ESI (ES+) mode.

Reaction kinetics

^{1}H-NMR spectra were recorded at 298K on a Bruker Avance III 600 spectrometer. The NMR sample contained 1mM PK11000 and 1mM glutathione in 25mM phosphate pH 7.2, 150mM NaCl, 1mM TCEP, and 5% D6-DMSO buffer. Aromatic proton peaks at 8.51ppm (adduct) and 8.93ppm (PK11000) were integrated to give concentrations of PK11000 and its GSH adduct over time. The data was then fitted with a second-order kinetics equation for equimolar adduct concentrations using Kaleidagraph (25):

$$c(product) = c_0 - \frac{1}{(k_2 * t + c_0)}$$

Fluorescence Polarization DNA Binding assay

5 μM stabilized full length p53 was incubated with 1mM compound for 2 hours at 4° C. The fluorescence polarization DNA binding assay was then performed, as described (52), in a 25mM sodium phosphate, 1mM TCEP, 150 mM sodium chloride, 5% DMSO assay buffer with a 5' fluorescein labelled GADD45 DNA response element (53). Data were fitted to the Hill equation including a linear drift term using Kaleidagraph.

Cell culture

H1299 cells were a kind gift from Carol Prives; H1299 cells with constitutively expressed p53 R175H were a kind gift of Fiona M. Townsley; and HCT116 p53-/- cell lines were a kind gift of Bert Vogelstein. HCT116 (wild-type p53), SW480 (p53-R273H/P309S) and SJSA-1 (wild-type p53) were purchased from ATCC; HUH-7(p53-Y220C+/+), HUH-6 (wild-type p53+/+). NUGC-3 (p53-Y220C+/+), NUGC-4 (wild-type p53+/+), and MKN1 (p53-V143A+/+) cells were obtained from the Japan Health Science Research Resources Bank and cultured as described.(11) Briefly, cells were maintained in DMEM (HUH-6, HUH-7, HCT116, SW480) or in RPMI 1640 (NUGC-3, NUGC-4, H1299, H1299 R175H, MKN1, SJSA-1) medium with 10% fetal calf serum and 1% antibiotic stock mix (10 000 U/ml penicillin, 10 000 mg/ml streptomycin) and incubated in a humidified incubator at 37 °C with 5% CO_2. The RPMI medium for culturing H1299 R175H was supplemented with 600 μg/ml G418.

Cell viability assay

Cell viability was measured using the CellTiter-Fluor cell viability assay kit (Promega, TB371) according to the instructions provided by the manufacturer. Cells were seeded in 96 well plates at 15000 cells per well and incubated overnight. Samples were prepared in medium with a twice as high compound and DMSO concentration then added to an equivalent volume of growth medium, yielding a final DMSO concentration of 0.5%. After incubating of cells for 23 hours or the respective time period, CellTiter-Fluor reagent was added to each well and incubated again for 45 minutes. Fluorescence was then recorded on a Pherastar plate reader (BMG Labtech, Germany) using 400/500-nm excitation/emission filters. Cell viability experiments were performed in quadruplicate and normalized with the values of blank (viability =1) and no cell (viability = 0) controls. Data are shown as mean ± SEM (standard error of the mean).

p53 Knockdown

p53 protein levels were down-regulated via transfection of human-specific p53 siRNA (Qiagen) using the INTERFERin siRNA Transfection Reagent (Polyplus). Negative control siRNA (Qiagen) was used as negative control for the siRNA transfection. The knockdown was confirmed by Western blots .

Western Blots

Cells were seeded in six-well plates at 0.5–0.8 million cells per Q:25 well and incubated overnight at 37 °C and 5% CO2. Cells were harvested after treatment with PK11007 at 0.5% DMSO per well for 3 h and lysed in RIPA buffer (Sigma-Aldrich) containing one cOmplete EDTA-Free Protease Inhibitor mixture tablet (Roche Diagnostics) per 50 mL RIPA buffer. Protein levels were determined using Coomassie Plus protein assay reagent (Thermo Scientific). SDS gel electrophoresis was conducted using NuPAGE 4–12% Bis-Tris gels (Life Technologies) loading 20 μg protein per lane. Proteins were electroblotted onto a Millipore Immobilon-P PVDF membrane (Millipore). Membranes were then blocked with PBS containing 5% dried skimmed milk for 1 h at RT, incubated with primary antibodies for 1 h (or overnight at 4 °C), and then with secondary antibodies coupled to horseradish peroxidase for 1 h. The blots were treated with GE ECL or ECL PRIME chemiluminescent detection reagent (Little Chalfont) and exposed to Fuji Super RX-N medical X-ray film for detection. The following antibodies were used: p53 (DO-1), p21 (187), GSTP1 (3F2C2), CHOP (sc-793), XBP-1 (sc-7160; Santa Cruz Biotechnology), PUMA (ab9643), β-actin (AC-15), MDM2 (2A10; Abcam). Anti–mouse-HRP (sc-2005) and anti–rabbit-HRP (sc-2004) antibodies were both

obtained from Santa Cruz Biotechnology.

Real-Time PCR

HUH-6, NUGC-3, HUH-7, and MKN1 cells were treated with PK11007 or DMSO control for 6 h (4.5 h for HUH-7). Total RNA was extracted and purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Synthesis of cDNA was performed with iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using the Rotor-Gene SYBR Green Kit (Qiagen) on a Rotor-Gene 6000 (Corbett Life Science) PCR cycler. The relative standard curve method was used to quantify relative mRNA levels. Each sample was measured in triplicate.

Caspase-3/7 and cytotoxicity assay

Caspase-3/7 activity and cytotoxicity were measured using the ApoTox-Glo Triplex assay (Promega). The assay was performed as described previously (54). Briefly, cells were incubated with compound or DMSO control for 6 hours. After 1 hour incubation with Caspase-Glo 3/7 reagent, luminescence was recorded using a Centro XS^3 LB 960 microplate luminometer (Berthold Technologies, Germany). All samples were measured in quadruplicate, with error bars depicting the standard error of the mean (S.E.M.). Significance levels were calculated using a one-way ANOVA with the Bonferroni post-hoc test for mean comparison (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

Detection of intracellular reactive oxygen species

Compounds and controls were added to yield a final sample DMSO concentration of 0.25%. After incubation for 1.5 hours at 37°C and 5% CO_2 and another 30 minutes after adding 0.5 μM CellROX Deep Red reagent (Life Technologies) and 1 μM SYTOX Blue Reagent (Life Technologies), the median CellROX Deep Red fluorescence of live cells was measured using an Eclipse Flow Cytometry Analyzer (Sony Biotechnology Inc.). Median fluorescence levels were determined in triplicates with error bars depicting SEM (Standard Error of Mean) values.

Example 1: Screening of fragment library and hit evaluation by NMR

An in-house fragment library was screened against stabilized full-length p53 (T-p53) using differential scanning fluorimetry (DSF) to identify ligands that increased the thermal stability of p53 (14, 15). The best hit, 5-chloro-2-methanesulfonylpyrimidine-4-carboxylic acid (PK11000), raised the protein melting temperature (T_m) by 1.5 K at 1 mM (Figure 1A). A similar stabilizing effect of PK11000 (ΔT_m > 1.2 K) was also found for the DNA-binding domain of the stabilized p53 cancer mutant Y220C (T-p53C-

Y220C) (16). $^{15}N/^{1}H$ HSQC NMR spectra of ^{15}N-labelled T-p53C-Y220C (94-312) with PK11000 concentrations ranging from 218 to 1000 μM confirmed that PK11000 binds to the DNA-binding domain (Figure 8. The observed peak shifts did not change in a concentration-dependent manner, indicating either slow or intermediate exchange-like behaviour or covalent modification. Interestingly, PK11000 mainly induced peak shifts of residues in direct proximity of Cys124, Cys182, and Cys277 (Figure 1B). The chemical shifts of several residues within the helix 1 and loop 1 region (including Cys124 and Cys277) were particularly large, indicating a strong effect of PK11000 binding on the chemical environment of this region (Figure 8). A less pronounced effect of PK11000 was observed for the region around Cys182.

The aggregation of p53 Y220C core domain at 37 °C, measured by light scattering, was inhibited by PK11000 at 250, 500, and 1000 μM to a similar extent (Figure 1C). This pattern was interpreted as being caused by a covalent modification of the protein during the time course. Amines have been reported to react with 2-sulfonylpyrimidines at high concentrations in dimethyl sulfoxide (17).

Example 2: Study of PK11000 alkylation of cysteine residues of p53

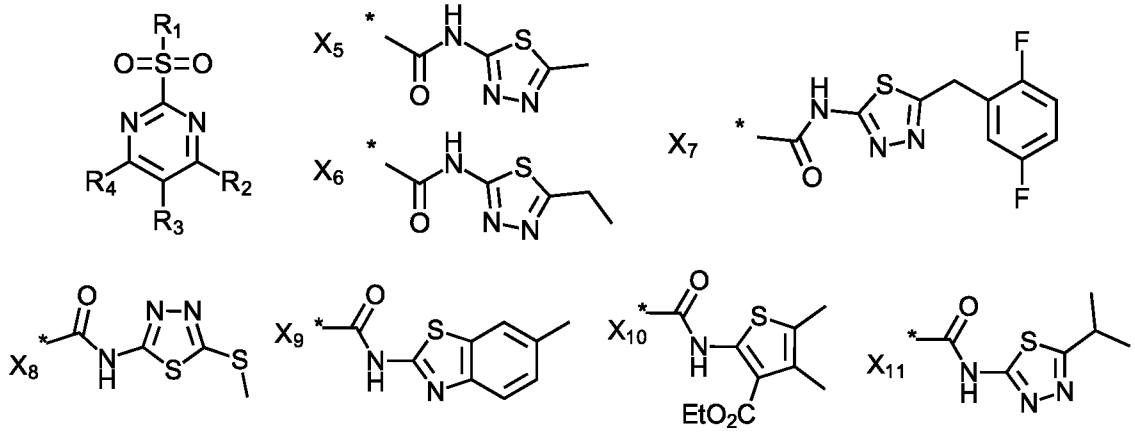
Covalent modification of cysteines in p53 was confirmed using ESI mass spectrometry experiments. A nucleophilic substitution reaction between PK11000 and a cysteine should lead to elimination of methyl sulfinic acid and an increase in the protein mass by 156.5 Da (Figure 2A). Incubation of 50 μM T-p53C-Y220C with various PK11000 concentrations for 4 hours at 20 °C yielded specific mass increases of either 157 Da or 2 x 157 Da (Figure 2B), confirming the proposed SNAr reaction mechanism. At low PK11000 concentrations (250 μM) with a 5:1 compound:protein ratio, we observed a mixture or mono and dialkylated proteins. For concentrations higher than 500 μM the maximum number of modified cysteines never exceeded two, even at a PK11000 concentration as high as 5 mM. Incubation of stabilized full-length p53 (T-p53) with 2 mM PK11000 also yielded exactly two modifications. T-p53C-Y220C contains 6 at least partly solvent-accessible cysteines (plus three zinc coordinating and two buried cysteines) (18). In contrast, the active form of APR-246, MQ, alkylates up to 5 cysteines of the R175H mutant at 2 mM concentration, and up to 9 cysteines with 5 mM MQ. This selectivity of PK11000 at neutral pH conditions for specific p53 cysteines suggests that 2-sulfonylpyrimidines react under biologically ambient conditions only with SNAr accessible and highly nucleophilic cysteines and are therefore useful tools for selective chemical protein modification.

Example 3: Study of effect on protein stability and DNA-binding affinity of specific modification of Cys182 and Cys277 by PK11000

HSQC NMR data suggested three potential candidates for alkylation by PK11000: Cys124, Cys182, and Cys277 (Figure 1). To determine which two of these three residues undergo alkylation, the effect of PK11000 on Cys-to-Ser mutants C124S/C182S, C124S/C277S, C182S/C277S, and C124S/C182S/C277S was monitored by electrospray ionization (ESI) mass spectrometry. Treatment with PK11000 yielded no modification for the triple mutant C124S/C182S/C277S and the double mutant C182S/C277S, but exactly one modification for the mutants C124S/C182S and C124S/C277S (Figure 3A), thus confirming specific alkylation of Cys182 and Cys277 by PK11000. Accordingly Cy182 and Cys277 are the most reactive nucleophiles on the p53 core for SNAr reactions with 2-sulfonylpyrimidines.

The mass spectrometry data were also in good agreement with DSF measurements of the effects of PK11000 on p53 protein stability (Table 1). While 30 min incubation of the p53 DBD with 250 μM PK11000 increased the melting temperature by about 3 °C, the compound had no effect on the stability of the C182S/C277S mutant. Comparison of the stabilizing effects of PK11000 on the C124S/C182S and C124S/C277S mutants further suggested that alkylation of Cys277 (ΔT_m = 3.6 K) has a stronger stabilizing effect than that of Cys182 (ΔT_m = 1.2 K).

Table 1 shows DSF ΔTm values of different p53 mutants after 30 minutes incubation with 250 μM of diverse 2-sulfonylpyrimidine compounds.



Wherein the * represents where the groups X_5-X_{11} are attached to the rest of the molecule.

Compound	R_1	R_2	R_3	R_4	ΔTm QMp53 DBD Y220C	ΔTm QMp53	ΔTm QMp53 DBD C124S/ C277S	ΔTm QMp53 DBD C124S/ C182S	ΔTm QMp53 DBD C182S/ C277S
PK11000	Me	COOH	Cl	H	2.9	2.3	1.2	3.6	0.0
PK11002	Me	COOH	Br	H	3.2	2.7	1.4	3.5	0.0
PK11003	Me	COOMe	Br	H	2.3	1.7	0.7	2.1	-0.2
PK11004	Me	COOEt	Br	H	2.1	1.5	0.6	1.4	-0.2
PK11005	Et	COOH	Cl	H	1.4	1.5	0.7	3.1	0.0
PK11006	Et	X_5	Cl	H	2.7	2.5	0.8	3.6	0.0
PK11007	(p-F)-Bn	X_5	Cl	H	3.5	3.0	0.9	3.6	-0.2
PK11008	(o-F)-Bn	COOH	Cl	H	0.2	0.0	0.0	-0.1	0.0
PK11009	(p-F)-Bn	COOH	Cl	H	2.5	2.2	1.3	3.7	0.1
PK11010	Me	X_6	Br	H	3.4	2.8	0.5	3.4	-0.2
PK11011	Me	H	OH	H	0.0	-0.1	0.0	-0.1	0.0
PK11012	Me	C(O)NHPh	Cl	H	1.1	0.7	-0.7	-0.2	-0.6
PK11013	Me	X_7	Cl	H	0.9	0.9	-0.5	0.6	0.1
PK11014	Me	C(O)NH (o-Br-p-Me-Ph)	Cl	H	-0.1	-0.5	-0.8	-1.6	-0.2
PK11015	Me	C(O)NH (m-Cl-p-Me-Ph)	Cl	H	-0.1	0.3	-2.0	-1.3	-1.2
PK11016	Me	COOH	$N(Bn)_2$	H	2.5	2.1	0.9	3.5	0.1
PK11017	Me	Me	H	Me	0.1	0.0	0.0	-0.2	0.3
PK11018	Me	CF_3	H	(o-OMe) -Bn	0.9	-0.5	0.1	-0.8	0.5
PK11029	Me	X_7	Cl	H	3.5	2.4	-	-	-
PK11038	(4-F)Bn	X_8	Cl	H	2.6	2.0	-	-	-
PK11039	(4-F)Bn	X_9	Cl	H	1.6	1.3	-	-	-
PK11042	(4-F)Bn	X_{10}	Cl	H	-0.3	-0.3	-	-	-
PK11043	(4-Me) Bn	X_5	Cl	H	3.0	2.5	-	-	-
PK11044	(4-F)Bn	X_6	Cl	H	3.1	2.3	-	-	-
PK11046	(4-F)Bn	Ph	Cl	H	1.1	0.1			
PK11047	Bn	X_5	Cl	H	3.1	2.3			
PK11048	(4-F)Bn	C(O)NH (4-F-Ph)	Cl	H	0.9	-0.2	-	-	-
PK11049	(4-F)Bn	X_{11}	Cl	H	3.0	2.3	-	-	-
PK11050	(4-F)Bn	C(O)NH (4-CO2Et-Ph)	Cl	H	0.6	-0.3	-	-	-

Upon binding of p53 to DNA response elements, Cys277 forms weak polar interactions with bases in the major groove of the target DNA (19, 20). Therefore a fluorescence polarization based DNA-p53 binding assay was performed using the GADD45a response element sequence to see whether alkylation of Cys277 with different p53

stabilizing 2-sulfonylpyrimidines interferes with DNA binding (Figure 9). Incubation of T-p53 with 1 mM PK11000 or PK11007 and PK11010, two structural analogs with larger ring substituents (see Table 1 for chemical formulas), for 2h had little effect on the p53-GADD45a K_d, which ranged from 26-36 nM compared to 31 nM for the untreated protein, therefore demonstrating that alkylation of p53 by 2-sulfonylpyrimidine derivatives does not alter its DNA-binding capability.

As a second example for alkylation of specific surface-exposed cysteines by 2-sulfonylpyrimidines, 50 μM of bacterial N-acetylneuraminate lyase was incubated with 2 mM PK11000. This tetrameric enzyme contains 4 cysteines of which one is located on the surface of the tetramer but not fully solvent exposed. In this case no chemical protein modification was observed, confirming that this compound modifies only specific cysteines residues within a protein, depending on nucleophilicity of the cysteine and geometrical constraints for transition state formation

Example 4: Structural effects of Cys182 modification

Crystals of Y220C DBD were soaked with PK11000 and the structure of the complex was determined at 1.6 Å resolution. Cys182 and Cys277 are freely accessible in the crystal lattice and after initial refinement of the model, the difference density map showed positive density at these cysteines. For Cys277 the density was not clear enough to unambiguously model the modification, whereas for Cys182 there was conclusive electron density for a chloropyrimidine moiety covalently linked to the sulphur (Figure 4). The carboxylate group was not well resolved, suggesting ring flipping. The modification forms only few interactions with the rest of the protein, primarily via His178. But interestingly, modification of Cys182 affects the conformational equilibrium of this region. In the unmodified form, Cys182 adopts two alternative conformations. Upon modification however only one side-chain conformation is observed, and the backbone is also fixed in a single conformation.

Example 5: Reactivity and p53 stabilizing effects of 2-sulfonylpyrimidine derivatives

The rate of reaction of PK11000 with glutathione (GSH) was measured by 1H-NMR spectroscopy at 20°C, using equimolar concentrations of reagents. The second-order rate constant was 1.37 [L mol-1 s-1] (Figure 5), 1000, 100, and 10 times lower, respectively than for the reported Michael acceptors, 1-penten-3-one, methyl propiolate, and methyl acrylate with GSH (21). The GSH reactivity of the acrylamide moiety, which is present in the FDA-approved anticancer drugs Ibrutinib and

Canertinib, is 7 times less reactive than PK11000 (22). Considering the clear correlation of compound-GSH reactivity with toxicity (21, 23, 24), the reactivity of the PK11000 is close to the level of therapeutically applied thiol reactive agents.

The effects of analogues of PK11000 (Figure 6A) on the thermal stability of p53 after 15, 30, 60 and 120 min incubation were then examined. Increasing stabilization of T-p53C-Y220C with PK11000 over time, rising to a maximum ΔT_m of about 2.5 K (Figure 6B) was observed. Substituting the 4-chloro group of PK11000 with bromine (PK11002) yielded slightly higher p53 T_m shifts of about 3 K after 60 minutes incubation. The largest T_m shifts (> 3K) were observed for PK11007 and PK11010, which also appeared to react faster than other 2-sulfonylpyrimidines, as they reached their maximum effect after only 15 minutes. These two compounds share an electron withdrawing 4-N-(5-methyl/ethyl-1,3,4-thiadiazol-2-yl)carboxamide substituent, which may increase the reaction rate. Interestingly, large, substituted acetanilide or 5-benzyl-thiadiazol-2-yl-carboxamide substituents on the pyrimidine scaffold of PK11013-15 resulted in a significant destabilization of p53 (Figure 6C). Compounds with electron donating groups, such as 3,5 dimethyl (PK11017) or 4-hydroxyl (PK11011) substituents, had no effect on p53 protein stability. Interestingly, 2-chloro-pyrimidines, which are reported to exhibit SNAr reactivity (26), had no significant effect on the p53 melting temperature. Overall, these clearly show the potential for fine-tuning the reactivity of the 2-sulfonylpyrimidine core scaffold through appropriate substitutions.

Example 6: Study of anti-cancer activity of 2-sulfonylpyrimidines

A study was carried out to determine whether 2-sulfonylpyrimdines exert biological effects on cancer cells. It is unlikely that biological effects in cancer cells are exclusively caused by modification of only one specific protein such as p53. A multitude of other thiol containing molecules, such as the abundant intracellular antioxidant glutathione (GSH) and proteins that possess sufficiently nucleophilic and surface accessible cysteines are likely to undergo modification, which may lead to a complex perturbation of various cellular processes and pathways.

To assess the anti-cancer activity of 2-sulfonylpyrimidines, cell viability of fivep53 wild-type cell lines (HUH-6, NUGC-4, SJSA-1, HCT-116, WI38) and eight p53 mutant cell lines (HUH-7, NUGC-3, SW480, SW620, SW1088, BXPC-3, MKN-1, H1299 R175H), and two p53 null cell lines (HCT-116 p53 -/-, H1299) was measured after 24 hours or 72 hours incubation with PK11007 (Table 2 contains a detailed description of the tested cancer cell lines). A significant viability reduction was observed in mutant p53 cell

lines MKN1 (V143A), HUH-6 (Y220C), NUGC-3 (Y220C), and SW480 (R273H/P309S) at concentrations ranging from 15 and 30 μM (Figure 7A). The p53 WT cell lines HUH-6 and NUGC-4 were significantly less sensitive to PK11007, and cell viability reduction was observed only at high compound concentrations (60 and 120 μM). Interestingly, the p53 WT cell line SJSA-1, which is known for its high intracellular MDM2 levels, was as sensitive to PK11007 as the tested mutant p53 cancer cells. A time-dependent analysis of PK11007 mediated HUH-6 and HUH-7 viability reduction revealed that the mutant p53 cells were not only more sensitive to PK11007 at lower concentrations, but also died more quickly than wild-type p53 cells (Figure 11). A stronger selectivity for the p53 mutant cell lines was also observed for PK11000 and PK11010 albeit only at higher compound concentrations.

Table 2 - Overview of cancer cell lines

cell line	p53 status	organism	tissue type	disease	ATCC/JCRB code
WI38	wt	human	lung	-	CCL-75
HUH-6	wt	human	liver	hepatoblastoma	JCRB0401
NUGC-4	wt	human	stomach	gastric adenocarcinoma	JCRB0834
SJSA-1	wt	human	fibroblast (lung)	osteosarcoma	CRL-2098
HCT-116	wt	human	colon	colorectal carcinoma	CCL-247
HCT-116 p53 -/-	knockout	human	colon	colorectal carcinoma	- Note 1
SW480	R273H-P309S	human	colon	Dukes' type B, colorectal adenocarcinoma	CCL-228
SW620	R273H-P309S	human	colon	Dukes' type B, colorectal adenocarcinoma	CCL-227
SW1088	R273C	human	brain	astrocytoma	HTB-12
HUH-7	Y220C	human	liver	hepato cellular carcinoma	JCRB0403
NUGC-3	Y220C	human	stomach	gastric adenocarcinoma	JCRB0822
MKN1	V143A	human	stomach	adenosquamous carcinoma	JCRB0252

BXPC-3	Y220C	human	pancreas	adenocarcinoma	CRL-1687
H1299 R175H	R175H	human	lung	non-small cell lung cancer	- Note 2
H1299	null	human	lung	non-small cell lung cancer	CRL-5803

Note 1 Established by Bert Vogelstein (John Hopkins).

Note 2 H1299 transfected with pcDNA expression vector containing p53-R175H.

Additionally, PK11007 was tested with the p53 null cell line H1299 and the p53 mutant cell line H1299 p53 R175H (Figure 7B). PK11007 induced a strong viability reduction in both cell lines, comparable to the sensitivity of HUH-7 and NUGC-3 cancer cells. The isogenic HCT116 and HCT116 p53 -/- cell lines showed comparable sensitivity to PK11007 treatment, suggesting a p53-independent mode of action for PK11007.

Interestingly, APR-246 did not prove to be as effective as PK11007 in inducing cell death for the cancer cell lines tested (Figure 10). Cell viability was significantly reduced only at high concentrations of APR-246 (≥ 60 μM). Contrary to previous reports that p53 mutant cell lines are more sensitive to APR-246 than wild-type p53 cell lines (4, 5), we did not observe a significant difference in sensitivity after 24 hours treatment.

To further assess the anti-cancer activity of 2-sulfonylpyrimidines, cell viability of three p53 wild-type cell lines (HUH-6, NUGC-4, SJSA-1), four p53 mutant cell lines (HUH-7, NUGC-3, SW480, MKN-1) and one normal fibroblast cell line (WI-38, non-cancer) was measured after 24 hours incubation with PK11000, PK11003, PK11007, PK11009, PK11010, PK11012, PK11015, PK11028 and PK11029. The results for each compound are shown in Figure 15. The strongest effects on cell viability were observed for PK11007 and PK11012. Mutant p53 cell lines were mostly significantly more sensitive for these compounds than p53-WT and non-cancer cell lines. This behaviour was also observed for PK11010 and PK11029, however only at higher concentrations. PK11003 lead to a strong viability decrease, however, it did not distinguish clearly between mutant and wild-type p53 cell lines.

Additionally, PK11007 was tested with the p53−/− cell line H1299 and with it containing p53 R175H (Figure 7B). PK11007 strongly reduced viability in both cell lines, comparable to the sensitivity of HUH-7 and NUGC-3 cancer cells. Compared with p53-WT–containing HCT116, the isogenic HCT116 p53−/− cell line was less sensitive (Figure 7B). Down-regulation of p53 protein levels via siRNA did not change PK11007-mediated viability reduction in HUH-6 and HUH-7 cells (Figure 25), which

reinforces that PK11007 can induce cell death independently of p53. In MKN1 cells (p53-V143A), however, there was a significant viability difference between control and p53 knockdown samples at 15 and 20 μM PK11007, indicating that cell death is partly dependent on mutant p53 in this cell line. APR-246 was not as effective as PK11007 in inducing cell death for the cancer cell lines tested (Figure 10). Cell viability was reduced only at high concentrations of APR-246 (≥60 μM). The WT and mutant p53 cell lines were similarly sensitive after 24-h treatment, unlike previous different sets of cells after longer treatment (4, 5).

The cell viability results observed for various compounds against various cell lines 24 hours after treatment are provided in Table 3

Table 3 – Cell viability – 24 h treatment

Cell Line	PK11007 IC50 [μM]	Apr-246 IC50 [μM]	PK11009 IC50 [μM]	PK11010 IC50 [μM]	PK11012 IC50 [μM]	PK11015 IC50 [μM]
WI-38	60	104		>120	19	19
HUH6	47	42		87	23	
NUGC4	50	56	> 120	63	42	52
HUH7	24	49		58	6	
NUGC3	23	61	> 120	49	17	19
MKN1	15			23	6	
SJSA1	26					
SW480	24			71		22
H1299	16					
H1299 H175	16					
HCT116	25					
HCT116 p53 -/-	29					

Table 3 – Cell viability – 24 h treatment (continued)

Cell Line	PK11029 IC50 [μM]	PK11028 IC50 [μM]	Pk11038 IC50 [μM]	PK11042 IC50 [μM]	Pk11047 IC50 [μM]	PK11049 IC50 [μM]
WI-38						
HUH6	> 120					
NUGC4	> 120	> 120	26	30	28	28
HUH7	> 120					
NUGC3	99	> 60	14	17	17	18
MKN1	57					
SJSA1						
SW480						
H1299						

H1299 H175						
HCT116						
HCT116 p53 -/-						

The cell viability results observed for various compounds against various cell lines 24 hours after treatment are provided in Table 4.

Table 4 – Cell viability – 72 h treatment

Cell Line	PK11007 IC50 [μM]	Apr-246 IC50 [μM]	PK11012 IC50 [μM]	PK11029 IC50 [μM]	PK11038 IC50 [μM]	PK11042 IC50 [μM]	PK11047 IC50 [μM]
WI-38	42	67		>>60	28	6	35
HUH6	30	27					
NUGC4	28						24
HUH7	10	9					15
NUGC3	23						19
MKN1	11			47	12	6	9
SW1088	43	67					
SW620	11	20	4				
SW480	12	21	12				
BXPC3	20	27	34				17

Example 7: Study of mode of action of PK11007 in cancer cells

To further investigate the mode of action of PK11007 in cancer cells, caspase activity and loss of membrane integrity after 6 hours incubation was measured. There was no significant activation of caspases 3 and 7 in HUH-6 and HUH-7 cells after treatment with 30 or 60 μM PK11007, suggesting that the previously observed viability reduction in these cell lines is not the result of caspase-mediated apoptosis (Figure 13A). APR-246 treatment did also not increase caspase activity in these cells. PK11007 treatment resulted in a severe loss of membrane integrity in mutant p53 HUH-7 cells but not in HUH-6 cells (Figure 13B). Interestingly, combination of 15 μM PK11007 and 100 μM buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, had a synergistic effect on cytotoxicity. APR-246 treatment did not increase membrane permeability in HUH-6 or HUH-7 cells, consistent with the relatively low viability reduction of APR-246 in these cell lines. PK11007 slightly increased caspase 3/7 activity in SW480 (p53 R273H/P309S), SJSA-1 (p53 wt), and HCT116 (p53 wt) cells at certain concentrations (Figure 13C), suggesting that the compound may kill cancer cells in a similar way as APR-246, PRIMA-1, or MIRA-3, which are known to activate caspases in various cancer cell lines (4, 13, 27, 28). Although PK11007 showed some increase in caspase 3 and caspase 7 activities in several cases, viability reduction of very sensitive cancer cell lines

such as HUH-7 or MKN1 appears to be caused mainly through caspase-independent pathways.

Example 8: Study of effect of combination of PK11007 and buthionine sulfoximine

Glutathione (GSH) is the major redox buffer in cells and is crucial for many enzymatic and non-enzymatic antioxidant reactions that decrease oxidative stress (e.g. ROS) and maintain the redox state of the cell (29). Because of its high abundance in the cell in the millimolar range and its freely accessible thiol group (30), GSH is a prime target for modification by selective thiol alkylators. Lambert et al. reported that APR-246 mediated growth suppression is potentiated by inhibition of glutathione synthesis via buthionine sulfoximine (BSO), an inhibitor of glutamate cysteine ligase (5). To assess whether the observed cell viability reduction for PK11007 is also enhanced by BSO, we incubated HUH-7, HUH-6, NUGC-3, NUGC-4, and MKN1 cell lines with 15 μM PK11007, 100 μM BSO, or a combination of both (Figure 7C). BSO treatment alone did not affect viability in any cell line. Combination of PK11007 and BSO resulted in a significantly stronger viability reduction in the mutant p53 cell lines MKN1, HUH-7 and NUGC-3 than PK11007 alone. This strong synergistic effect was not observed in WT p53 HUH-6 and NUGC-4 cells.

Example 9: Study of the effects of PK11007 on p53 and p21 protein levels in HUH-6 (p53-WT) and HUH-7 (p53-Y220C) cells.

The effect of different concentrations of PK11007 on p53 and p21 protein levels in HUH-6 (p53-WT) and HUH-7 (p53-Y220C) cells was investigated. It was found that PK11007 induced no significant change in p53 levels in HUH-6 (see Figure 14). At higher concentrations (30 and 60 μM), PK1007 lead to a small mass increase of p53 (circa 4 kDa), which suggests that p53 becomes hyperalkylated at this compound concentration. p21, which transcription is mainly regulated by p53, could only be detected for HUH-6 cells at the used settings. PK11007 up-regulated p21 protein levels in a concentration dependent way, suggesting that transcription of p53 target genes may be increased via PK11007 in HUH-6 cells. The Western blot shows that in p53 WT cells (HUH-6), p53 target genes (shown in this figure for p21) are upregulated upon PK11007 treatment. This may explain the decreased sensitivity of p53 wild-type cells for PK11007, as p53 activation leads also to upregulation of several important antioxidant genes such as GSTP1 and SESN1 that could help to detoxify ROS in the cell.

Example 10: Study of effect of PK11007 on ROS levels

Recent studies suggest that the biological effects of APR-246 are not directly dependent on the p53 status but rather on the perturbation of the intracellular GSH/ROS (reactive oxygen species) balance and the respective cellular context (31-33). To test whether PK11007 also increases ROS levels, NUGC-3, NUGC-4, HUH-6, HUH-7 and MKN1 cells were incubated with PK11007 for 2 hours and stained the cells with CellROX Deep Red dye, which exhibits a strong fluorescent signal upon oxidation by ROS. Elevated ROS levels were found in all cell lines after 2 h. In the mutant p53 cells MKN1, HUH-7 and NUGC-3, however, the ROS increase was significantly higher at 60 μM PK11007 than in NUGC-4 and HUH-6 cells, suggesting that the higher PK11007 sensitivity (and faster viability reduction) of the mutant p53 cell lines is mediated by a stronger ROS induction (Figure 7D). Interestingly, basal and PK11007 induced ROS levels in MKN1 cells were significantly higher than in other cell lines, which may have contributed to the strong induction of cell death at low PK11007 concentrations in this cell line (Figure 7A). Treatment of HUH-6 and HUH-7 cells with N-acetylcysteine (NAC) slightly reduced ROS levels of untreated cells (Figure 12A), which is in line with its well-known anti-oxidative effect (34). NAC also prevented PK11007-mediated ROS increase in both HUH-6 and HUH-7 cells. This effect was most likely not only caused by the antioxidant effect of NAC, but also the result of adduct formation with the alkylating agent, as shown for APR-246 (5).

Changes in cellular ROS levels can alter expression of many genes, activate cell signalling cascades and induce apoptotic or necrotic cell death at high intracellular levels (29, 35). The relatively fast induction of ROS and viability reduction upon PK11007 treatment strongly suggests that compound-mediated ROS increase is the main mechanism for the observed cell death. The strong synergism of PK11007 and BSO further suggests that the observed ROS increase is due to depletion of intracellular glutathione levels via GSH-PK11007 adduct formation. Similar effects were also found for piperlongumine, a Michael-acceptor containing natural-product that selectively kills cancer cells (36).

Interestingly, PK11007 induced ROS often more effectively in mutant p53 cancer cell lines, which may be the reason for stronger and potentially faster viability reduction in NUGC-3, HUH-7 and MKN1 cells. The p53 WT cell line SJSA-1, however, was as sensitive to PK11007 treatment as the tested mutant p53 cell lines. Functional inactivation of p53 via high intracellular MDM2 levels could be one factor for the increased sensitivity of this cell line. While promoting pro-oxidant and apoptotic pathways at very high stress levels, p53 exerts pro-survival and antioxidant responses

at modest or transiently elevated oxidative stress levels (29). ROS directly increase p53 activity (29, 37), which leads to upregulation of several genes with anti-oxidative effects, including glutathione peroxidase 1 (GPX1) and mitochondrial superoxide dismutase 2 (SOD2) (38) or phosphate-activated mitochondrial glutaminase (GLS2), which is an important enzyme for providing L-glutamate as substrate for GSH synthesis (39). Increased ROS levels and oxidation of DNA bases were observed upon knockdown of p53, in cancer cells with mutant p53, or cancer cells with elevated MDM2 levels (38), which further reinforces that wild-type p53 cells may possess an important protection mechanism against toxic ROS levels.

Interestingly, there was no significant difference in viability reduction between the isogenic cell lines HCT116 and HCT116 p53 -/-, which lacks expression of full-length p53 (40), suggesting that cellular effects of PK11007 are independent of p53 in this cell line. However, the remarkable sensitivity of the HCT116 cell lines for PK11007 may be caused by different factors. According to the catalogue of somatic mutations in cancer (COSMIC) in the canSAR database (41, 42), HCT116 contains several missense or frameshift mutations in important anti-oxidative genes such as thioredoxin reductase 1 (TXNRD1), which is also inhibited by APR-246 (43), and other thioredoxin containing proteins, glutathione S-transferase alpha 2 (GSTA2), and glutathione S-transferase P (GSTP1) (see Table 5 for a detailed description of gene mutations). Potential defects in the cellular ROS detoxification system may explain the high sensitivity for PK11007 and the missing protection from oxidative stress via wild-type p53 in HCT116 cells.

Table 5 - Somatic mutations in anti-oxidative genes

Protein family		NUGC-3	NUGC-4	HUH-7	HUH-6	SJSA-1	MKN1	SW48	H1299	HCT116
Glutathione peroxidases	GPX	-	-	-	-	-	-	-	-	GPX4 (c.325-2A>G)[c]
Glutathione S-transferases	GST	-	-	-	GSTK1 (p.M100I)	-	GSTM2 (p.N59S)	-	-	GSTA2 (c.273-3C>T; c.249G>T)[c]; GSTP1 (G208V)
Thioredoxin containing proteins	TXN	-	-	-	TXNDC11 (p.N440N; p.N467N)	-	-	-	-	TXN2 (p.K152N); TXNDC5 (p.F368S); TXNDC12 (p.L7L); TXNRD1 (c.1_2insT; c.451_452insT)[c]
Peroxi-redoxins	PRDX		-	-	-	-	-	-	-	-
Superoxide dismutases	SOD	-	-	-	-	-	-	-	-	-
Catalase	CAT	-	-	-	-	-	-	-	-	-
Gluta-redoxins	GLRX	-	-	-	-	-	-	-	-	-

Glutamate-cysteine ligase	GCL	-	-	-	-	-	-	-	-	-
Gluta-minase	GLS2	-	-	-	-	-	-	-	-	-
Glutathione synthetase	GSS	-	-	-	-	-	-	-	-	-
Gene mutation data		2237[a]	889[a]	932[a]	1036[a]	785[a]	774[a]	16[b]	145[b]	5100[a]

[a] Data taken from COSMIC – Cell Lines Project; [b] data from COSMIC database; [c] mutation 'syntax' follows the HGVS nomenclature recommendations (55)

[a] Data taken from COSMIC – Cell Lines Project; [b] data from COSMIC database; [c] mutation 'syntax' follows the HGVS nomenclature recommendations (55)

Taken together, these findings suggest that PK11007 and other thiol modifying compounds, such as PRIMA, MIRA, and STIMA, despite alkylating and stabilizing the p53 protein, exert their antitumor function not necessarily via reactivating p53, but via other cellular mechanisms, such as increase of cellular ROS to toxic levels (33). This would also explain the seemingly contradictory reports that PRIMA, MIRA-1, and STIMA-1 induce cell death also in cell lines with DNA- contact mutants that cannot be rescued by simple protein stabilization (5, 13, 27).

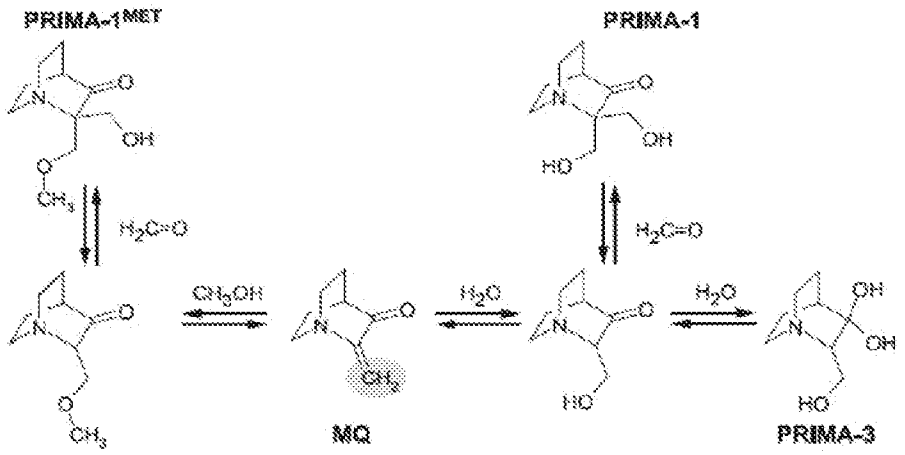
Example 11: Study of anti-breast cancer activity of 2-sulfonylpyrimidines

Three of the compounds PK11000, PK11007 PK11010 were tested against a panel of breast cancer cell lines including several triple negative, HER2+, luminal and immortalised breast epithelial cell lines.

Triple negative breast cancer refers to any breast cancer where the three most common types of receptor known to fuel most breast cancer growth (i.e. estrogen, progesterone and HER-2/neu gene) ae not present in the cancer tumour. This means that the breast cancer cells have tested negative for hormone epidermal growth factor receptor 2 (HER-2), estrogen receptors (ER), and progesterone receptors (PR). This make triple negative breast cancer a challenging target that is difficult to treat because most chemotherapies target one of these three common types of receptor. Triple negative breast cancer occurs in approximately 15% of diagnosed breast cancers.

For comparison, PRIMA-1 and PRIMA-1^{MET} were also tested against the same panel of breast cancer cell lines. PRIMA-1 and PRIMA-1^{MET} are two compounds that can restore wild-type properties to mutant p53 and such compounds are potentially most useful in cancers with a high prevalence of p53 mutations. Presently, early clinical trials are ongoing evaluating the anti-mutant p53 agent, PRIMA-1^{MET}. The structures of these

two compounds and the interconversions that may take place between them are shown below (5).



Cell Culture

The following panel of breast cancer cell lines were used including Hs578T(i8), MDA-MD-468, HCC1937, HCC1143, BT20, BT549, (all TN), Sum 159 , MDA-MD-453, SKBR3, BT474, JimT1, UACC-812 (all HER2 amplified), T47D, ZR-75-1, MCF7 (all luminal), MCF12A and MCF10A (immortalised breast epithelial). Hs578t(i8) cells which were supplied by Dr Susan McDonnell, University College, Dublin; this cell line was derived from the parental Hs578t cell line by sequential selection through *in vitro* invasive chambers (Hughes, L., et al., Clin Exp Metastasis, 2008. 25(5): p. 549-57). All other cell lines were purchased from the American Type Culture Collection (ATCC). Cell lines were maintained through continued passaging at 37°C with a humidified atmosphere of 5% CO_2. All media was supplemented with 10% foetal bovine serum (FBS) (Invitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies) and 1% Fungizone (Invitrogen Life Technologies). BT549 cells were maintained in RPMI 1640 supplemented with 0.023 IU Insulin (Sigma-Aldrich), 10 mM Hepes (Sigma-Aldrich), 1.5 g/L sodium bicarbonate (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich). ZR-75-1 cells were maintained in DMEM supplemented with 1 nM Estradiol (Sigma-LDRICH). UACC812 cells were maintained in Lebovitz L-15 media. MCF10A and MCF12A cells were maintained as per reference (60). All other cell lines were maintained in RPMI 1640. Cell line identity was confirmed by analysis of Short Term Repeat Loci (IdentiCell, Denmark) and cells were routinely tested for mycoplasma infection.

Cell viability assay

Cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich). To test the effect of PK11000, PK11007

and PK11010 on proliferation, cells were plated at a density of 1×10^3/well in 96-well flat-bottomed plates (Sigma-Aldrich). Following overnight incubation, quadruplicate wells were treated with a range of concentrations of compounds ranging from 0 to 50 μM. After 5 days, 0.5 mg/ml MTT was added to each well and incubated at 37 °C for 5 hr. Media was aspirated, and 200 μL of DMSO (Santa Cruz Biotechnology) was added to each well, for 5 min. Absorbance was measured at a wavelength of 550 nm on a microplate reader (Multiscan Ascent, Labsystems).

Table 6 - shows the mean IC50 results for treatment with each of these compounds PK11000, PK11007, PK11010, PRIMA-1 and PRIMA-1^{MET} against the panel of breast cancer cell lines.

			IC_{50} value (μM)				
Cell Line	**Subform**	**p53 status****	**PK 11000**	**PK 11007**	**PK 11010**	**PRIMA-1**	**1PRIMA-1MET**
HCC1143	TN	p.Arg248Gln	4.98 ± 1.72	2.33 ± 0.3	19.03 ± 1.72	9.86 ± 2.1	6.81 ± 1.
HCC1937	TN	p.Arg306X	11.7 ± 1.8	6.75 ± 3.2	>50	4.73 ± 0.6	6.04 ± 1.2
BT20	TN	p.Lys132Gln	10.91 ± 3.2	11.77 ± 2.8	>50	5.86 ± 0.6	4.18 ± 0.4
Hs578T(i8)	TN	p.Val157Phe	16.54 ± 5.6	5.26 ± 0.7	25.32 ± 2.5	15.13 ± 2.8	11.91 ± 2.3
MDA-MD-468	TN	p.Arg273His	18.78 ± 1.8	2.77 ± 0.3	23.89 ± 1.0	2.49 ± 0.5	1.93 ± 0.3
MDA-MD-453	TN	p.His368delinsGln	>50	6.91 ± 3.2	>50	1.42 ± 0.3	0.90 ± 0.2
BT549	TN	p.Arg249Ser	5.39 ± 0.9	3.36 ± 0.6	13.77 ± 1.1	4.95 ± 0.2	3.11 ± 0.4
Sum 159	Her2+	p.Arg273His	>50	6.77 ± 1.3	>50	3.47 ± 0.7	3.29 ± 0.5
SKBR3	Her2+	p.Arg175His	4.09 ± 1.1	3.14 ± 0.9	15.43 ± 1.9	10.39 ± 2.5	5.13 ± 0.6
UACC812	Her2+	Wild Type	>50	42.23 ± 7.2	>50	5.60 ± 0.7	6.36 ± 1.5
BT474	Her2+	p.Glu285Lys	>50	21.2 ± 1.5	>50	3.97 ± 2.7	3.59 ± 1.1
JimT1	Her2+	p.Arg248Trp	11.4 ± 2.3	10.82 ± 1.1	47.35 ± 8.1	12.53 ± 1.4	11.26 ± 1.8
T47D	Luminal	p.Leu194Phe	18.71 ± 1.1	9.57 ± 1.1	30.24 ± 6.8	5.26 ± 0.6	3.25 ± 0.2
ZR751	Luminal	Wild type	>50	18.36 ± 2.8	38.21 ± 3.2	5.79 ± 1.9	8.41 ± 4.9
MCF7	Luminal	Wild Type	12.02 ± 5.5	13.17 ± 4.3	>50	11.10 ± 3.2	31.06 ± 24.6
MCF12A	Epithelial*	Wild Type	2.87 ± 1.2	18.63 ± 3.0	42.25 ± 11.6	7.68 ± 2.2	5.35 ± 1.6
MCF10A	Epithelial*	Wild Type	2.47 ± 0.7	16.52 ± 4.2	37.43 ± 9.5	8.66 ± 1.2	5.16 ± 1.3

*=Immortalised
** status obtained from literature
TN = Triple Negative

The mean IC_{50}± SEM were calculated using CalcuSyn™ software following MTT assay. All experiments were carried out in triplicate.

The results against various of the cell lines for each of the three compounds evaluated (Pk11007, Pk11000, and PK11010) are also shown in Figures 16-19.

It can be seen from these results that the three compounds (Pk11007, Pk11000, and PK11010) had an impact on the cell viability of a range of the breast cancer cell lines, in particular, on the triple negative subtype of breast cancer cells. The triple negative

subtype of breast cancer cells was especially sensitive to PK11007 which gave better results than either PRIMA-1 or PRIMA-1^{MET} for several cell lines.

Relationship between PK compounds and PRIMA-1 & PRIMA-1^{MET}

The statistical relationship between the IC_{50} values observed for the three PK compounds and the comparison compounds PRIMA-1 or PRIMA-1^{MET} was examined (see Table 7 below).

Table 7 - summarises the results of this statistical analysis.

	PK11007	**PK11010**	**PRIMA-1**	**PRIMA-1^{MET}**
PK11000	p=0.0743 r=0.4438	p=0.0481* r=0.4856	p=0.0287* r= -0.5299	p=0.3984 r= -0.2190
PK11007	N/A	p=0.0299* r=0.5267	p=0.7167 r=-0.09506	p=0.7454 r=0.08510
PK11010	p=0.0299* r=0.5267	N/A	p=0.1288 r=-0.3833	p=0.5511 r=0.1555

($p < 0.05$)*

The relationship between PK11007 and PRIMA-1^{MET} is shown visually in detail in Figure 22.

<u>Example 12: Study of effects of p53 knockdown on cell line response</u>

Lentiviral transfection

HEK293T packaging cells were seeded in a 6 well plate to 70 % confluence and incubated at 37 °C. Following overnight incubation they were treated with HEPEs buffer containing 25 mM chloroquine, 2.5 M CaCl2, PAX8 (envelope vector), VSVG (packaging vector) and pLKO.1 scrambled or pLKO.1 shp53 (Human) for 6 hr. The media was removed and stored at 4 °C. Fresh media was added to the cell and incubated at 37 °C for 48 hr. The media was removed and combined with the previous media at 4 °C. From this, the lentivirus was harvested using at 0.45 µM syringe. A 1:5 dilution of lentiviral:fresh media was added to the breast cancer cell line (MDA-MB-453) at 60 % confluence in a 6-well plate and incubated at 37 °C for 24 hr. Cells were washed with PBS, fed and incubated for a further 48 hr. Transfected cells were selected using 2 µM puromycin for three days. Knockdown was confirmed by qPCR.

The effects of p53 knockdown on cell line response was measured and the IC_{50} values for each of the compounds (Pk11007, Pk11000, and PK11010) along with two comparison compounds (PRIMA-1 or PRIMA-1^{MET}) are given in Table 8.

Table 8 – Shows the effects of p53 knockdown on cell line response.

		IC50 value (μM)				
Cell type	**p53 status**	**PK11000**	**PK11007**	**PK11010**	**PRIMA-1**	**PRIMA-1^{MET}**
Parental	p.His368delinsGln	>50	10.27 ± 2.9	>50	1.43 ± 0.3	0.90 ± 0.2
Scrambled	p.His368delinsGln	41.29 ± 6.2	2.68 ± 0.8	>50	2.97 ± 0.5	1.39 ± 0.6
p53 KO	p.His368delinsGln	>50	3.1 ± 1.4	>50	1.86 ± 0.8	1.21 ± 0.5

The effects of p53 knockdown are also shown in Figure 23 (for the comparison compound PRIMA-1^{MET}) and Figure 24 (for PK11007). In Figures 23 and 24 the abbreviations PAR, SCR and KO represent the results from parental, scrambled and knockdown cell types respectively.

Example 12: Study of effect of 2-sulfonyl pyrimidines on p53 target genes and on the unfolded protein response pathway

PK11007 up-regulated protein levels of the p53 target genes p21, MDM2, and PUMA in a mostly concentration-dependent manner in NUGC-3 (p53-Y220C), HUH-7 (p53-Y220C) and MKN1 (p53-V143A) cells, suggesting partial restoration of transcriptional activity to destabilized p53 mutants (Figure 26). PK11007 up-regulated protein levels of the p53 target genes p21, MDM2, and PUMA in HUH-6 and NUGC-4 (both p53 WT) cells. The molecular weight of blotted p53 gradually increased by ~3 kDa at 30 and 60 μM PK11007 in all mutant p53 cell lines, probably from alkylation of 11 residues in its denatured state (11 × 255 ~ 2.8 kDa). In contrast, neither the molecular weight nor level of p53 changed in the WT p53 cell lines HUH-6 and NUGC-4, indicating a smaller amount of alkylated, denatured p53 and possibly a higher tolerance against the thiol reactivity of PK11007. The asterisks in Figure 26 at the p53 target gene levels of HUH-6 and HUH-7 cells highlight the genes for which a different β-actin control was used.

Levels of GST P (GSTP1), an important enzyme for the detoxification of hydrophobic electrophiles via conjugation with glutathione, were slightly higher in the p53 WT NUGC-4 cells than in the mutant p53 NUGC-3 cells and may be one factor for the higher resistance against thiol reactive compounds in p53 WT cell lines. PK11007 also increased p53 activity in HUH-6 and NUGC-4 cells, as indicated by the increase of MDM2, PUMA, and p21 protein levels. At high concentrations, we observed in some cases a decrease in p53 target gene levels (e.g., p21 levels for NUGC-3 and MKN1), despite up-regulation of the same protein at lower concentrations. PK11007 increased

transcription of p53 target genes in three mutant p53 cell lines after 6-h treatment (Figure 27).

PUMA and p21 mRNA levels were up-regulated by a factor of 2 upon treatment of NUGC-3 (20 μM PK11007), MKN1 (15 μM), and HUH-7 (15 μM) cells, as well as NOXA for the latter two. The cells were treated with PK11007 for 6 h (4.5 h for HUH-7). The observed p53 mRNA levels did not change, except for a slight increase in HUH-7 cells. MDM2 levels were halved in MKN1 and NUGC-3 cells. There was no significant change in p53 target gene mRNA levels for the p53 WT cell line HUH-6 (20 μM PK11007).

Additionally, we observed activation of the unfolded protein response (UPR) pathway, which is triggered by endoplasmic reticulum (ER) stress. This was achieved by checking UPR key markers in MKN1, HUH-6, and HUH-7 cells after PK11007 treatment for 3 h (6 h for MKN1). PK11007 up-regulated protein levels of spliced XBP-1, a key marker for UPR activation, in a concentration- dependent manner in HUH-7 cells and to a lesser degree also in HUH-6 cells. At 20 μM PK11007, spliced XBP-1 was also up-regulated in MKN1 cells (Figure 28).

A similar observation was made for APR-246, which induced transcription of XBP-1 and splicing of its mRNA in Saos-2 and Saos-2-His273 cancer cells (61). Expression of CHOP, another UPR marker, was also increased by PK11007, especially in HUH-7 cells.

Conclusions

2-sulfonylpyrimidines have been identified as a biologically active class of selective thiol alkylators. They react rapidly and selectively with free thiol groups in neutral aqueous buffers at room temperature, allowing the introduction of specific molecular probes at solvent-exposed cysteines. Reactivity of the scaffold can be further fine-tuned by diverse substitutions that modify the electron density of the aromatic ring. Ultimately, thiol modification with diversely substituted pyrimidines expands the chemical space of the available toolkit for selective cysteine modification.

PK11007 and other 2-sulfonylpyrmidines were found to stabilize the DNA-binding domain of p53 via specific alkylation of the surface-exposed cysteines Cys182 and Cys277 without affecting its DNA-binding affinity. For example, PK11007 exhibited promising anti-cancer activity in various cancer cell lines (e.g. the hepatocellular carcinoma cell line HUH-7, the gastric cancer cell line NUGC-3, the adenosquamous carcinoma cell line MKN1, and the breast cancer cell lines HCC1143, Hs578T(i8) and

SKBR3). Its cell-death inducing effects appear to be glutathione-dependent and associated with strong ROS induction. This mechanism appears to be shared with the anticancer agent APR-246, which is currently in clinical trials, and may be the main mode of action thiol-reactive compounds with antitumor activity. PK11007 and derivatives represent promising anticancer drugs, in particular, for targeting cancer cells with non-functional p53 or impaired ROS detoxification systems.

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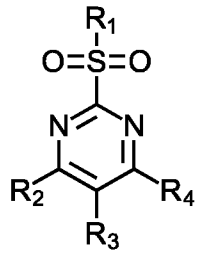
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All publications mentioned in the above specification are herein incorporated by reference. Although illustrative embodiments of the invention have been disclosed in detail herein, with reference to the accompanying drawings, it is understood that the invention is not limited to the precise embodiment and that various changes and modifications can be effected therein by one skilled in the art without departing from the scope of the invention as defined by the appended claims and their equivalents.

CLAIMS

1. A 2-sulfonylpyrimidine compound of formula (I):



(I)

and salts and solvates thereof for use in the treatment of a proliferative disease; wherein:

R_1 is selected from C_{1-6} alkyl, C_{6-12} aryl, C_{7-18} aralkyl, 6- to 15-membered heteroaralkyl and 6- to 12-membered heterocyclylalkyl wherein each of these groups are optionally substituted with from one to three optional substituents independently selected from methyl, ethyl, CH_2F, CHF_2, CF_3, CN, NO_2, OH, CO_2H, $CONH_2$, $C(O)NH(C_{1-6}$ alkyl), F, Cl, Br and I;

R_2 is selected from CF_3, O-C(O)-R_6, $C(O)R_7$, $C(O)NHR_7$ and $NHC(O)R_7$;

R_3 is selected from H, F, Cl, Br, I, OH, C_{1-6} alkyl, OC_{1-6} alkyl, CH_2F, CHF_2, CF_3, CN, NO_2, CO_2R_8, $C(O)NHR_{10}$, $NHC(O)R_{10}$, $(CH_2)_z$-NR_8R_9 and $(CH_2)_z$-NH-C(=NH)-NH_2;

R_4 is selected from H, C_{1-6} alkyl and CH_2R_{11};

R_5 is selected from C_{1-6} alkyl;

R_6 is selected from H and C_{1-6} alkyl;

R_7 is selected from 5- to 9-membered heteroaryl groups, and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, SR_5, CO_2R_5, F, Cl, Br, I, NO_2, OH, benzyl, fluorobenzyl and difluorobenzyl; and wherein the heteroaryl group is attached to the rest of compound of formula (I) by a carbon ring atom;

R_8 and R_9 are independently selected from H, C_{1-6} alkyl and benzyl;

R_{10} is selected from 5- to 9-membered heteroaryl groups, 5- and 6-membered heterocyclyl, and phenyl wherein these groups are optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, F, Cl, Br, I, NO_2, OH, benzyl, fluorobenzyl and difluorobenzyl;

R_{11} is phenyl optionally substituted with from 1 to 3 optional substituents independently selected from $NR_{12}R_{13}$ and OR_{12};

R_{12} and R_{13} are independently selected from H and C_{1-6} alkyl; and

z is selected from an integer selected from 0 to 6.

2. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to claim 1, wherein R_1 is selected from methyl, ethyl, phenyl, benzyl, para-fluoro-benzyl and ortho-fluoro-benzyl.

3. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein R_2 is $C(O)NHR_7$.

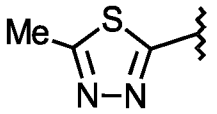
4. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein R_3 is selected from H, Cl, Br, OH and $N(CH_2Ph)_2$.

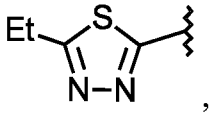
5. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein R_3 is selected from Cl and Br.

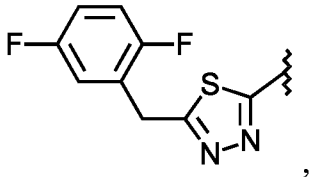
6. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein R_4 is selected from H, methyl and CH_2R_{11}.

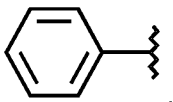
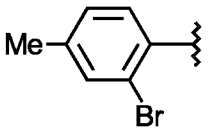
7. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein R_7 is selected from thiophenyl, 1,3,4-thiadiazolyl, benzothiazole and phenyl optionally substituted with from one to three optional substituents independently selected from C_{1-6} alkyl, SR_5, CO_2R_5, F, Cl, Br, I, benzyl, fluorobenzyl and difluorobenzyl.

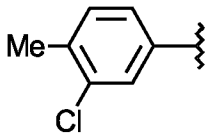
8. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein R_7 is selected from:



,


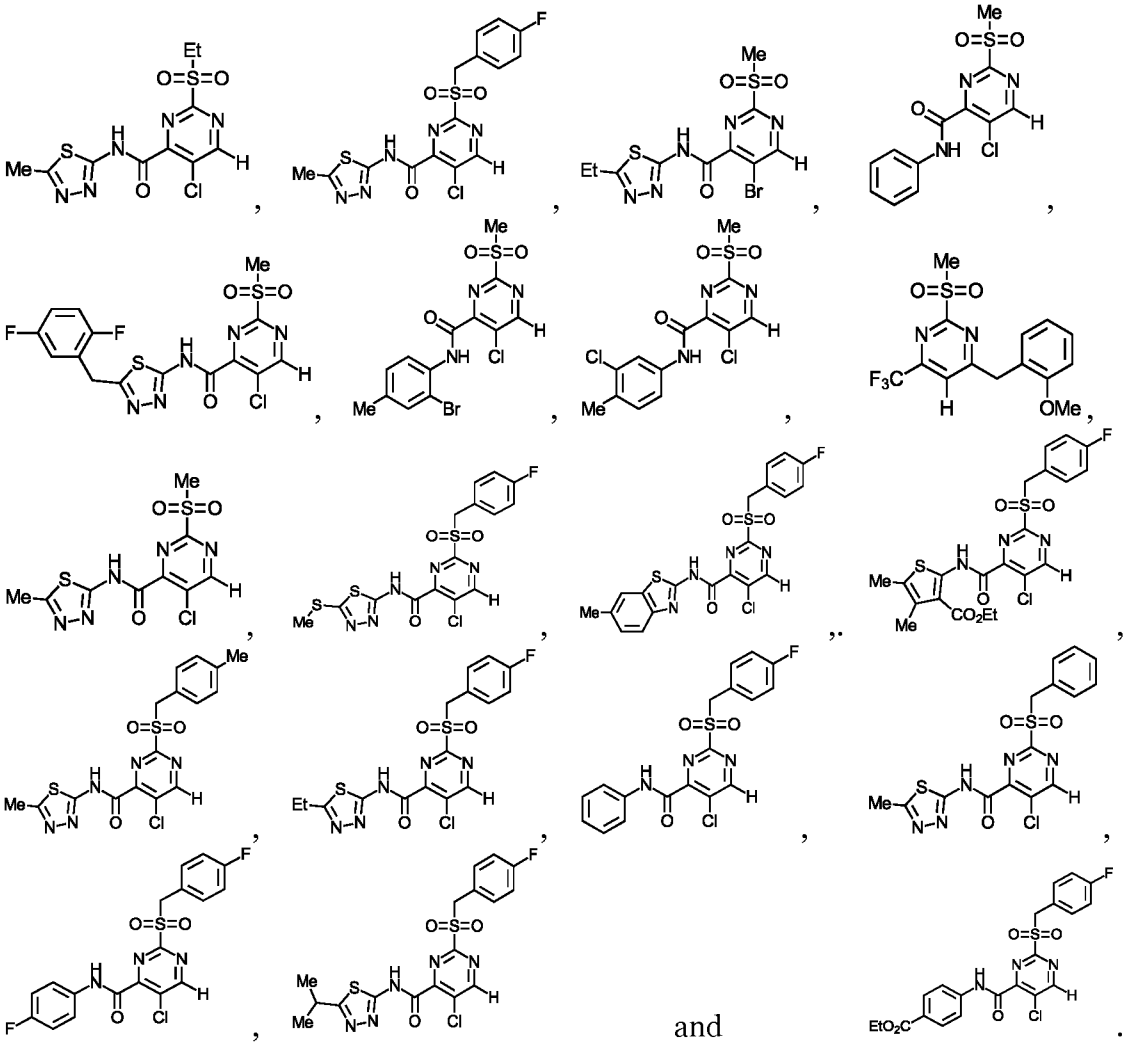
,


,

,


and


.

9. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein the compound of formula (I) is selected from:



and

10. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, wherein the proliferative disease is selected from bone cancer, breast cancer, colon cancer, liver cancer, lung cancer and stomach cancer.

11. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any of the preceding claims, for use in the treatment of a proliferative disease, wherein the 2-sulfonylpyrimidine compound is administered, either simultaneously or sequentially, with an inhibitor of glutamate cysteine ligase.

12. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to claim 11, wherein the inhibitor of glutamate cysteine ligase is buthionine sulfoximine.

13. A pharmaceutical composition comprising a 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof as defined in any one of claims 1 to 9, and an inhibitor of glutamate cysteine ligase.

14. A pharmaceutical composition according to claim 13, wherein the inhibitor of glutamate cysteine ligase is buthionine sulfoximine.

15. A 2-sulfonylpyrimidine compound of formula (I) and salts and solvates thereof according to any one of claims 1 to 12, for use in the treatment of a proliferative disease, wherein the 2-sulfonylpyrimidine compound is administered, either simultaneously or sequentially, with one or more pharmacologically active compounds and salts and solvates thereof suitable for treating anti-proliferative diseases selected from alkylating agents, platinum compounds, DNA altering compounds, microtubule modifiers, antimetabolites, anticancer antibodies, small molecule kinase inhibitors, drug conjugates, miscellaneous antitumor agents and mixtures thereof.

Figure 1

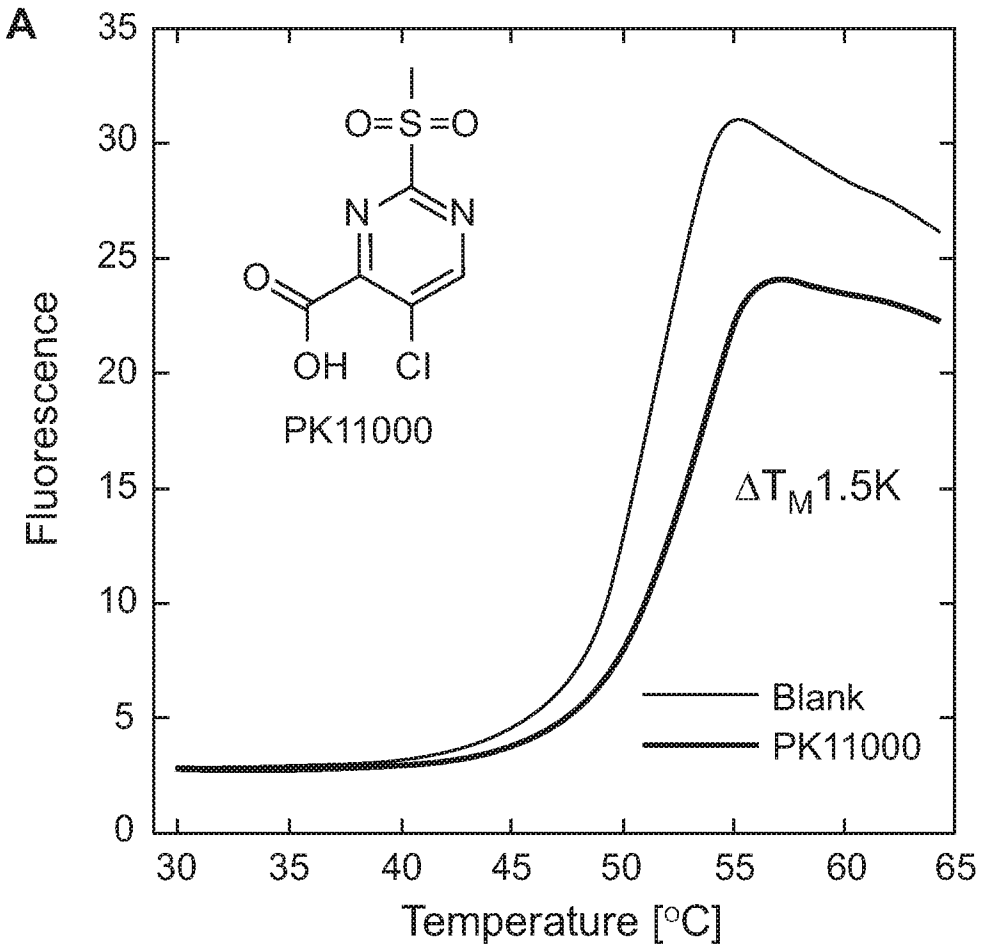

A
Fluorescence
Temperature [°C]
PK11000
ΔT_M1.5K
Blank
PK11000
B
R280
C277
C182
C124

Figure 1 continued

C

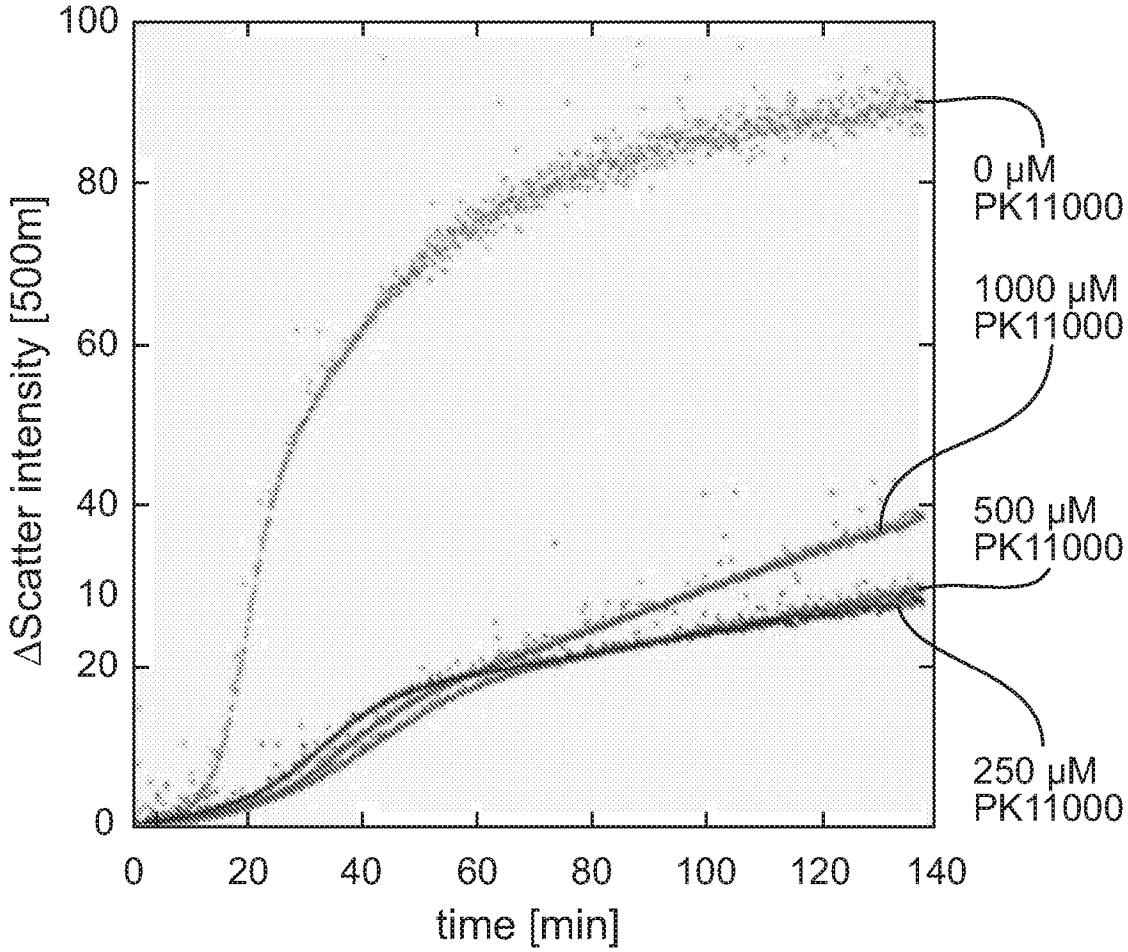


Figure 2

A

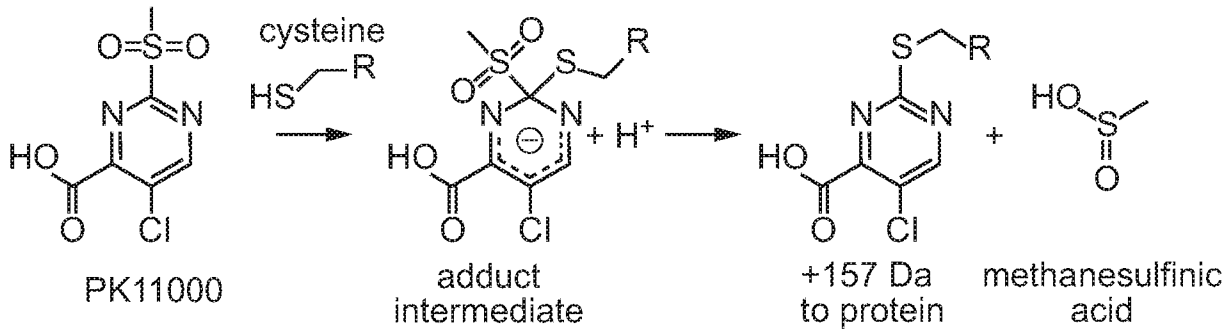


Figure 2

B

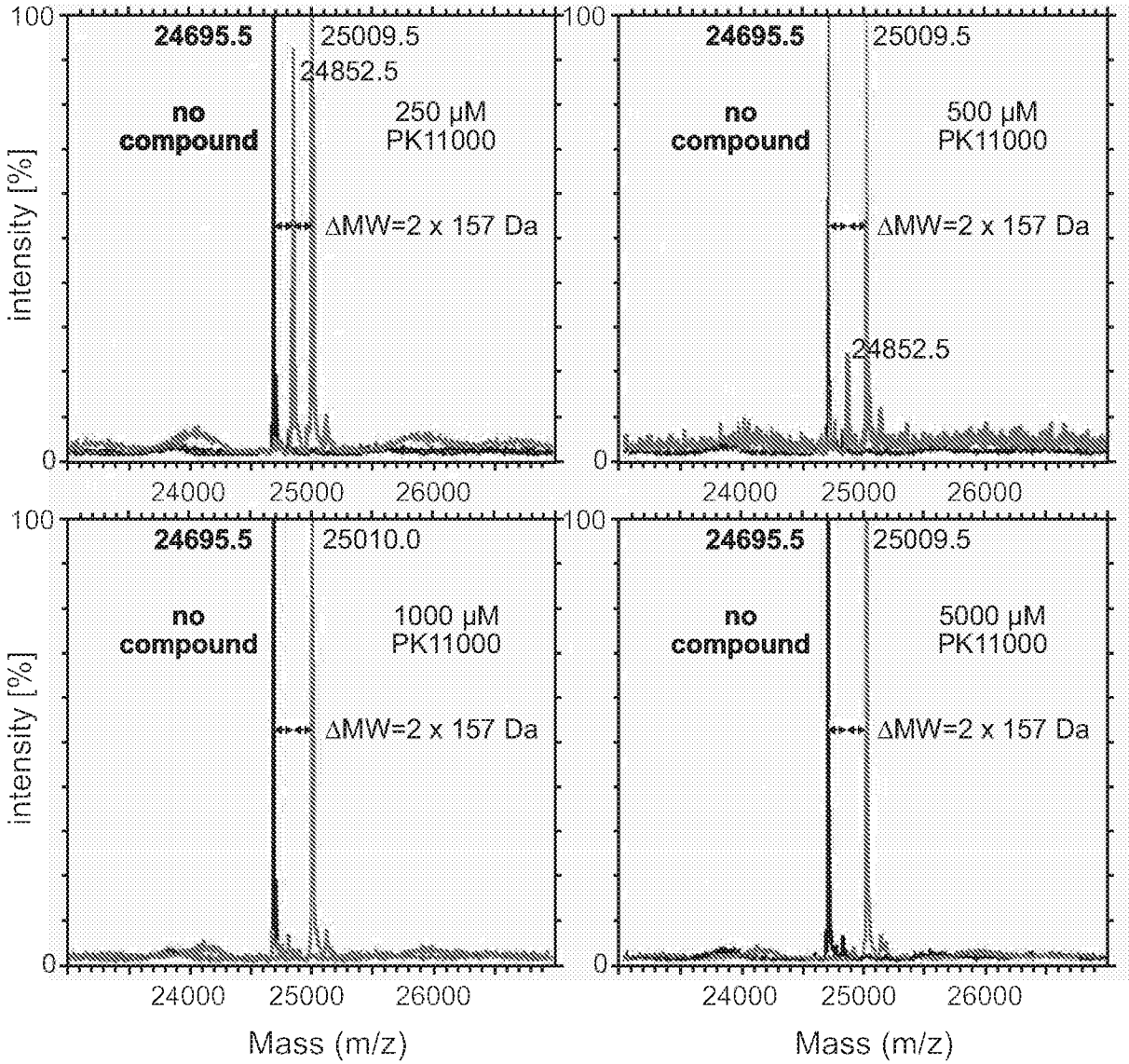

100
24695.5
25009.5
24852.5
no compound
250 µM PK11000
ΔMW=2 x 157 Da
intensity [%]
0
24000
25000
26000
100
24695.5
25009.5
no compound
500 µM PK11000
ΔMW=2 x 157 Da
24852.5
0
24000
25000
26000
100
24695.5
25010.0
no compound
1000 µM PK11000
ΔMW=2 x 157 Da
intensity [%]
0
24000
25000
26000
Mass (m/z)
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24000
25000
26000
Mass (m/z)

Figure 3

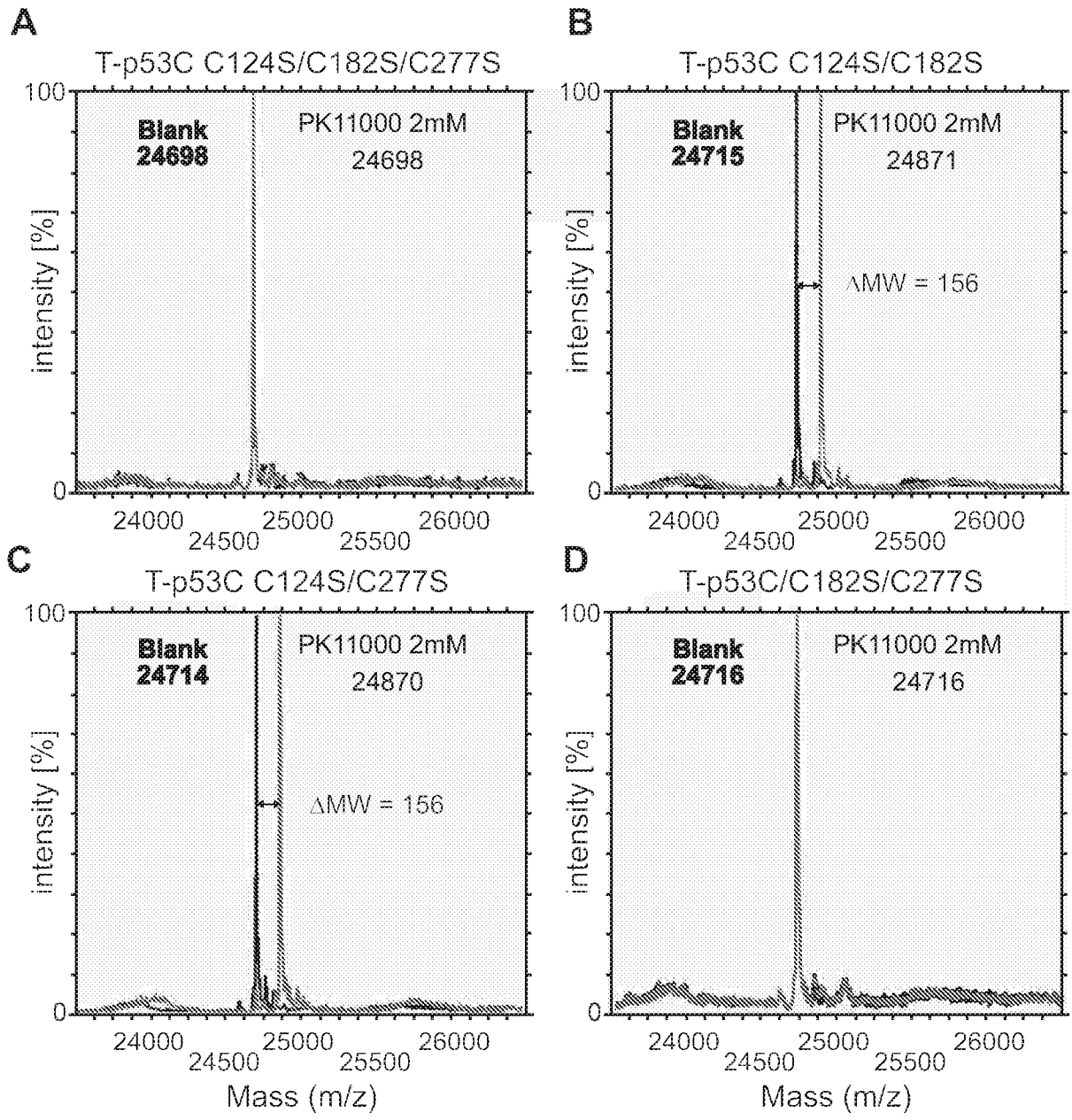

A
T-p53C C124S/C182S/C277S
Blank
24698
PK11000 2mM
24698
intensity [%]
100
0
24000
24500
25000
25500
26000
B
T-p53C C124S/C182S
Blank
24715
PK11000 2mM
24871
ΔMW = 156
C
T-p53C C124S/C277S
Blank
24714
PK11000 2mM
24870
ΔMW = 156
D
T-p53C/C182S/C277S
Blank
24716
PK11000 2mM
24716
Mass (m/z)

Figure 4

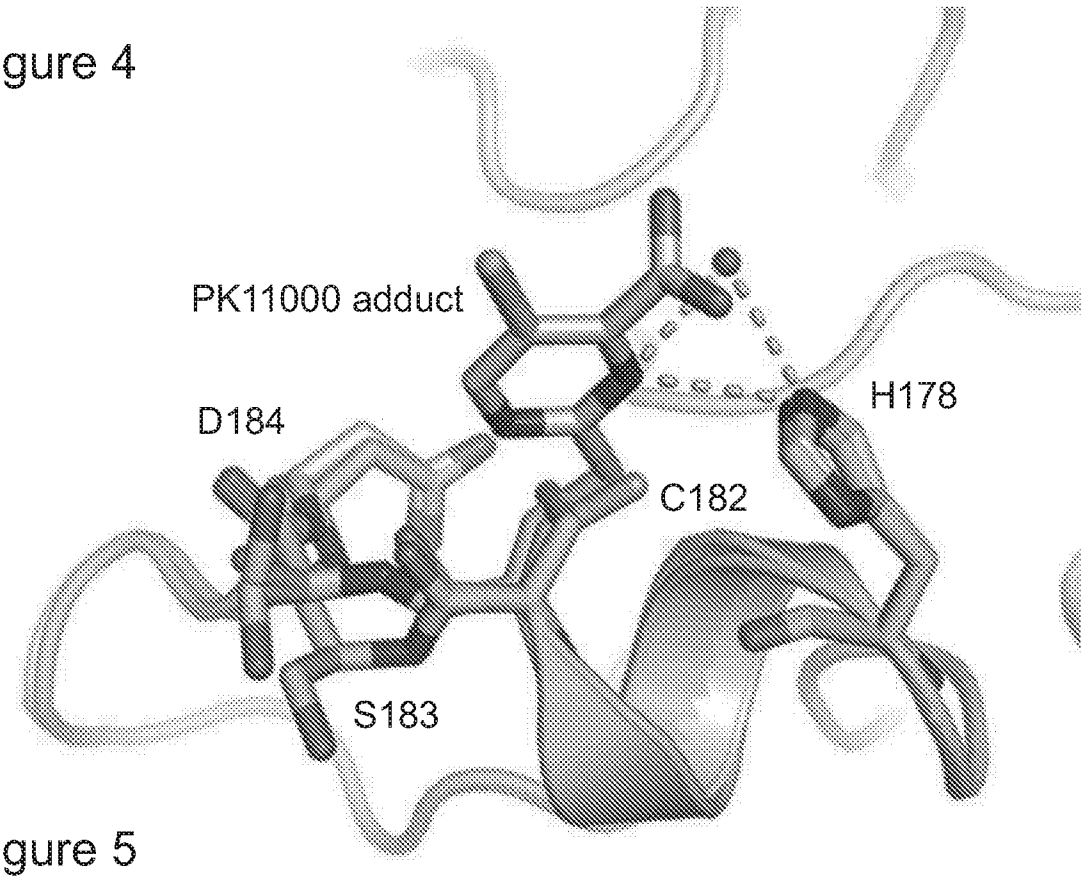


Figure 5

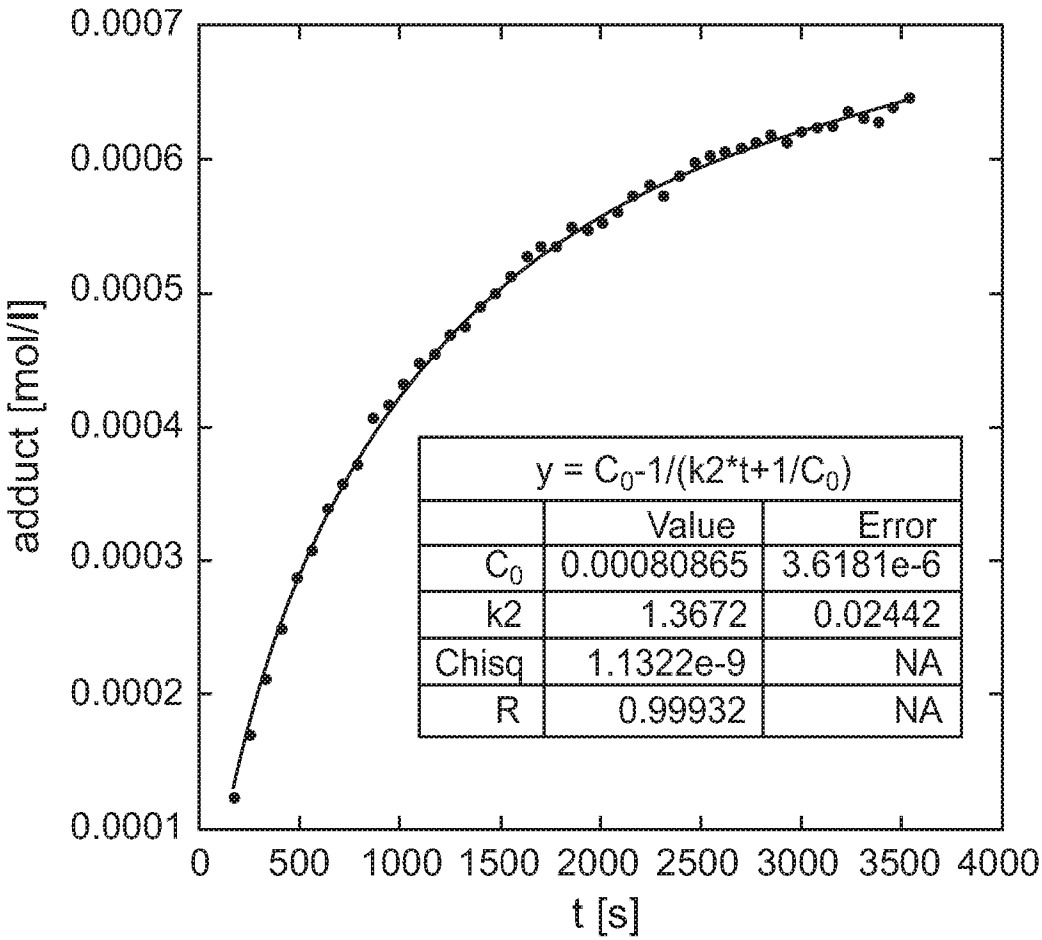
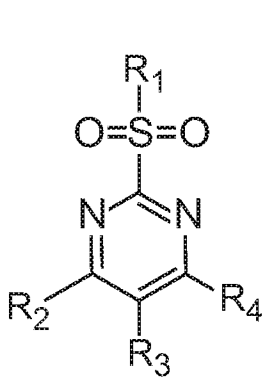
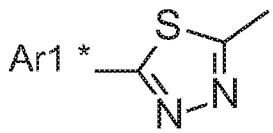


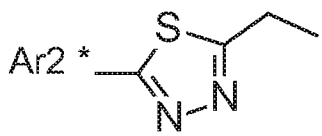
Figure 6

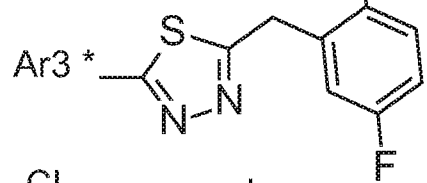
A

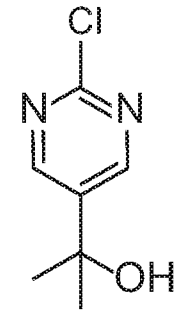
	RI	R2	R3	R4
PK11000	Me	COOH	Cl	H
PK11002	Me	COOH	Br	H
PK11003	Me	COOMe	Br	H
PK11004	Me	COOEt	Br	H
PK11005	Et	COOH	Cl	H
PK11006	Et	C(O)NH(**Ar1**)	Cl	H
PK11007	(4-F)-Bn	C(O)NH(**Ar1**)	Cl	H
PK11008	(4-F)-Bn	COOH	Cl	H
PK11009	(2-F)-Bn	COOH	Cl	H
PK110010	Me	C(O)NH(**Ar2**)	Br	H
PK110011	Me	H	OH	H
PK110012	Me	C(O)NH(Ph)	Cl	H
PK110013	Me	C(O)NH(**Ar3**)	Cl	H
PK110014	Me	C(O)NH(2-Br,4-Me-Ph)	Cl	H
PK110015	Me	C(O)NH(3-Cl,4-Me-Ph)	Cl	H
PK110016	Me	COOH	$N(Bn)_2$	H
PK110017	Me	Me	H	Me
PK110018	Me	CF_3	H	(2-OMe)-Bn
PK110028	Me	$C(O)NH_2$	Cl	Me
PK110029	Me	C(O)NH(**Ar1**)	Cl	H



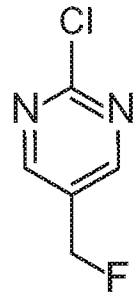




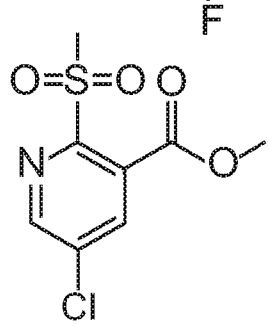




PK11024



PK11025



PK11026

Figure 6

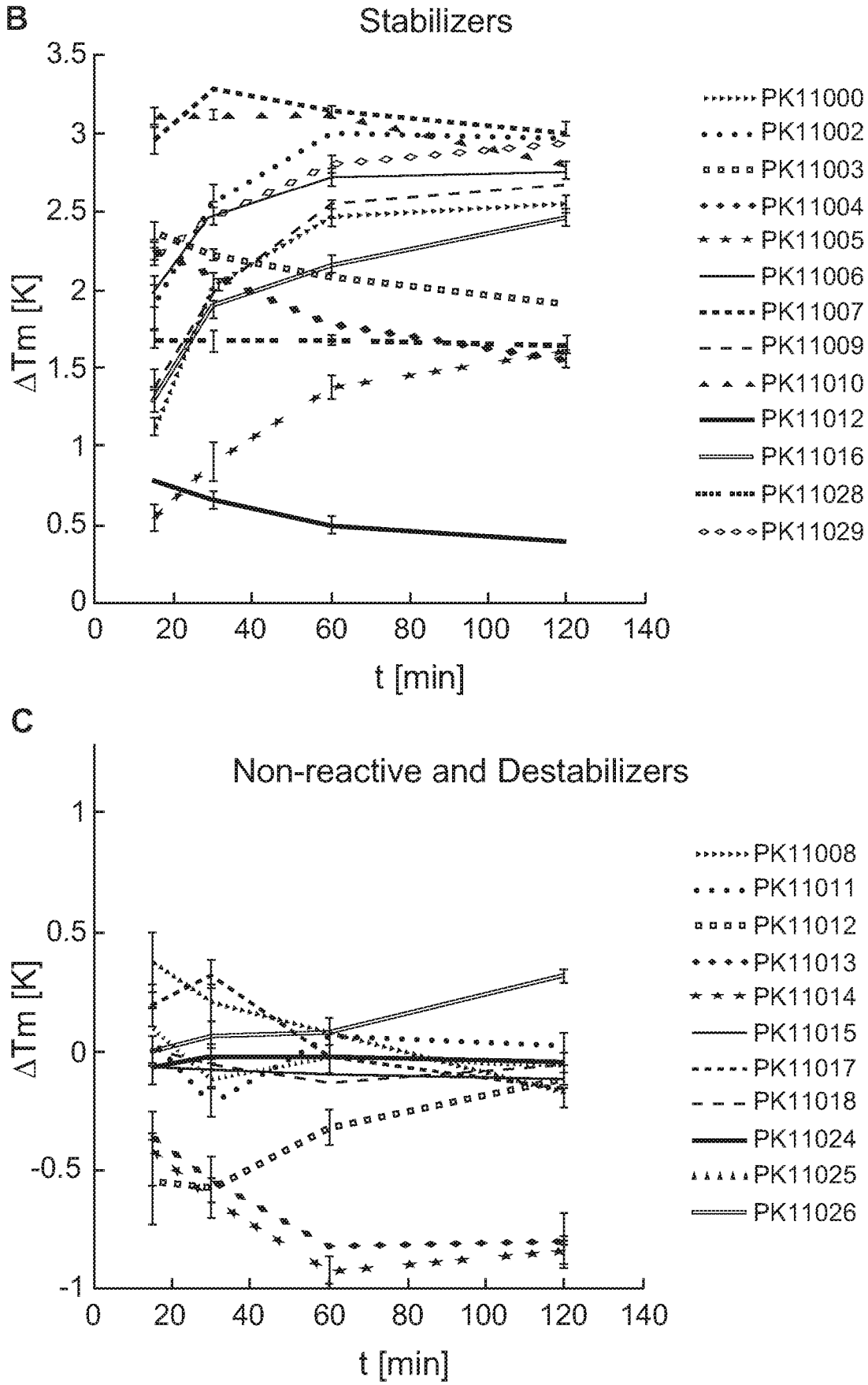

B
Stabilizers
ΔTm [K]
t [min]
PK11000
PK11002
PK11003
PK11004
PK11005
PK11006
PK11007
PK11009
PK11010
PK11012
PK11016
PK11028
PK11029
C
Non-reactive and Destabilizers
ΔTm [K]
t [min]
PK11008
PK11011
PK11012
PK11013
PK11014
PK11015
PK11017
PK11018
PK11024
PK11025
PK11026

Figure 7

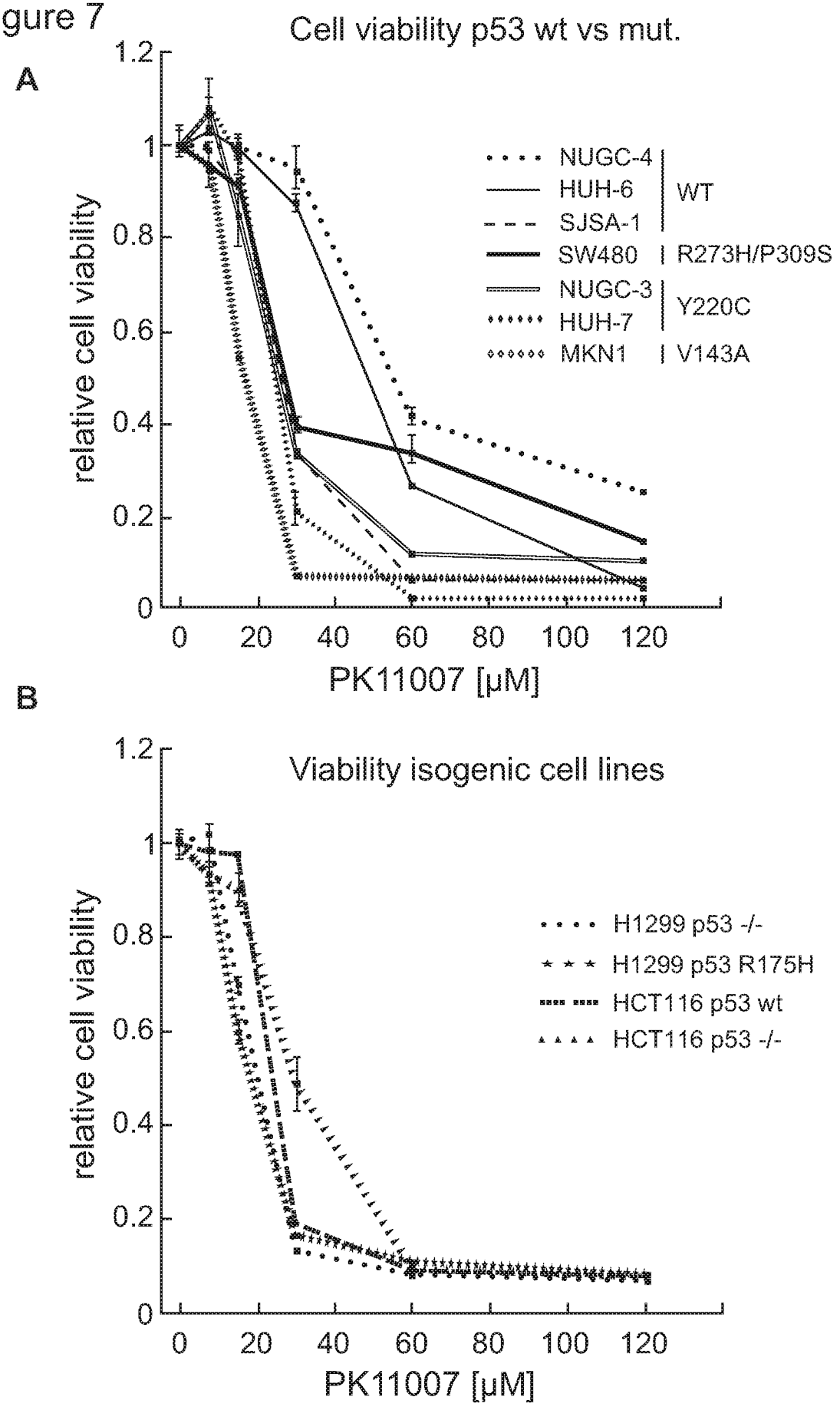
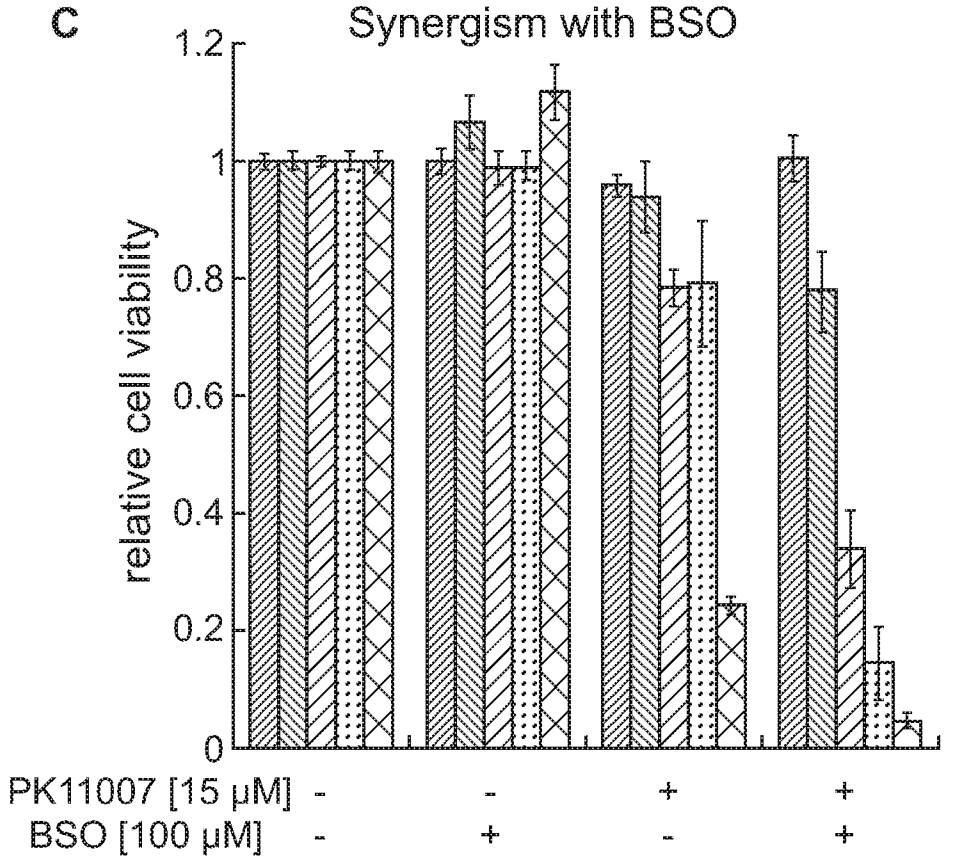

A
Cell viability p53 wt vs mut.
relative cell viability
PK11007 [µM]
NUGC-4
HUH-6
SJSA-1
SW480
NUGC-3
HUH-7
MKN1
WT
R273H/P309S
Y220C
V143A
B
Viability isogenic cell lines
relative cell viability
PK11007 [µM]
H1299 p53 -/-
H1299 p53 R175H
HCT116 p53 wt
HCT116 p53 -/-

Figure 7 continued



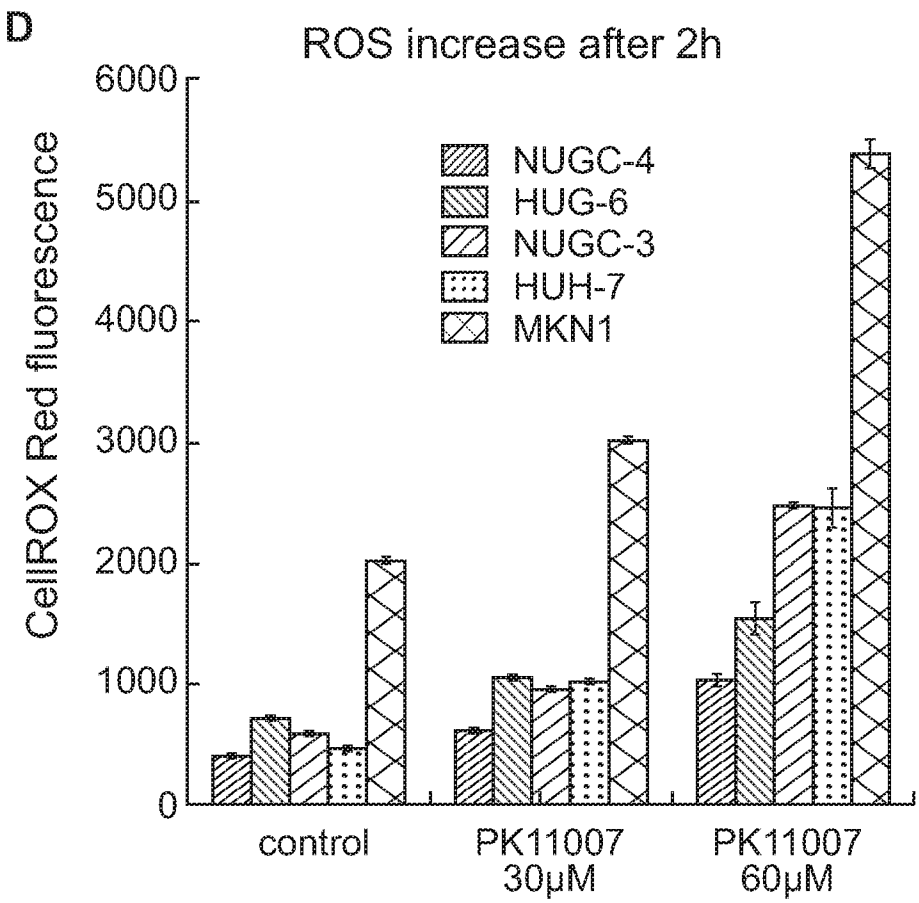
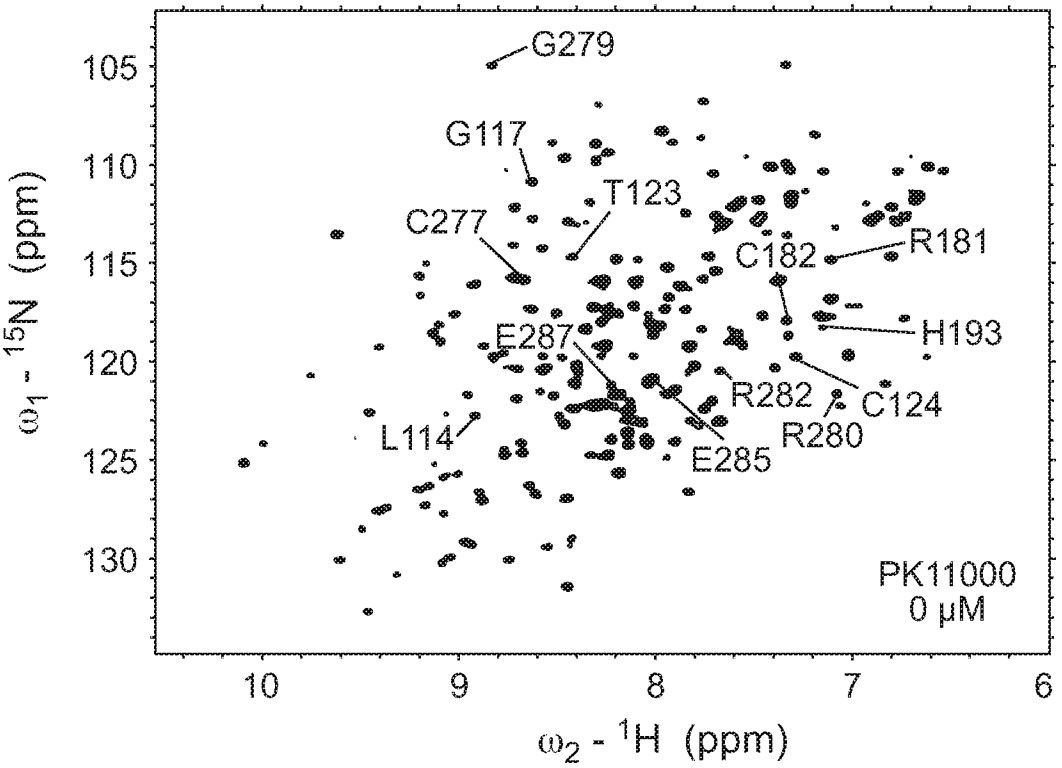


Figure 8


G279
G117
T123
C277
C182
R181
H193
E287
R282
C124
L114
R280
E285
PK11000
0 µM
105
110
115
120
125
130
10
9
8
7
6
ω1 - 15N (ppm)
ω2 - 1H (ppm)

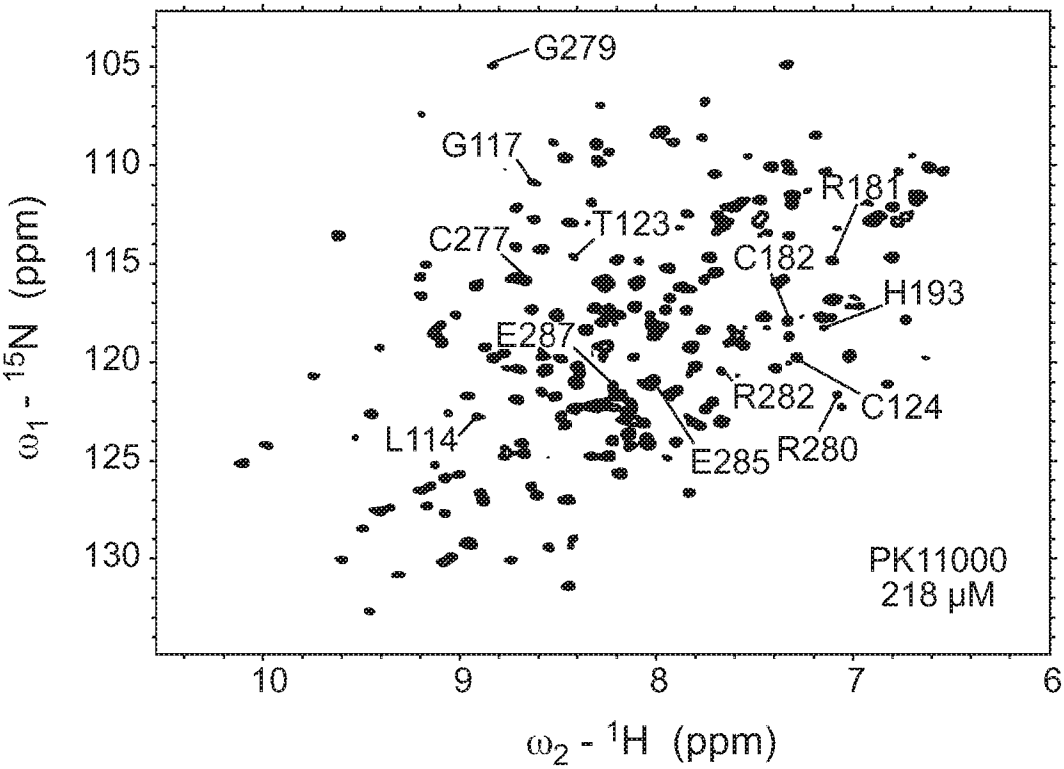
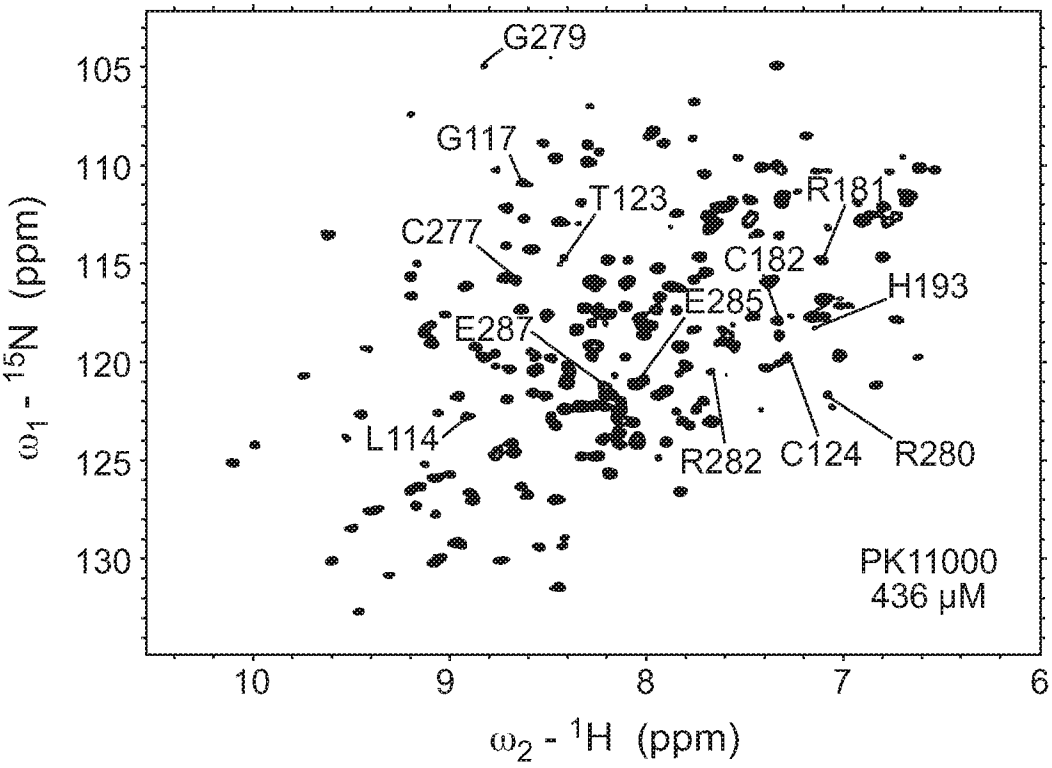

G279
G117
R181
T123
C277
C182
H193
E287
R282
C124
L114
E285
R280
PK11000
218 µM
105
110
115
120
125
130
10
9
8
7
6
ω1 - 15N (ppm)
ω2 - 1H (ppm)

Figure 8 continued



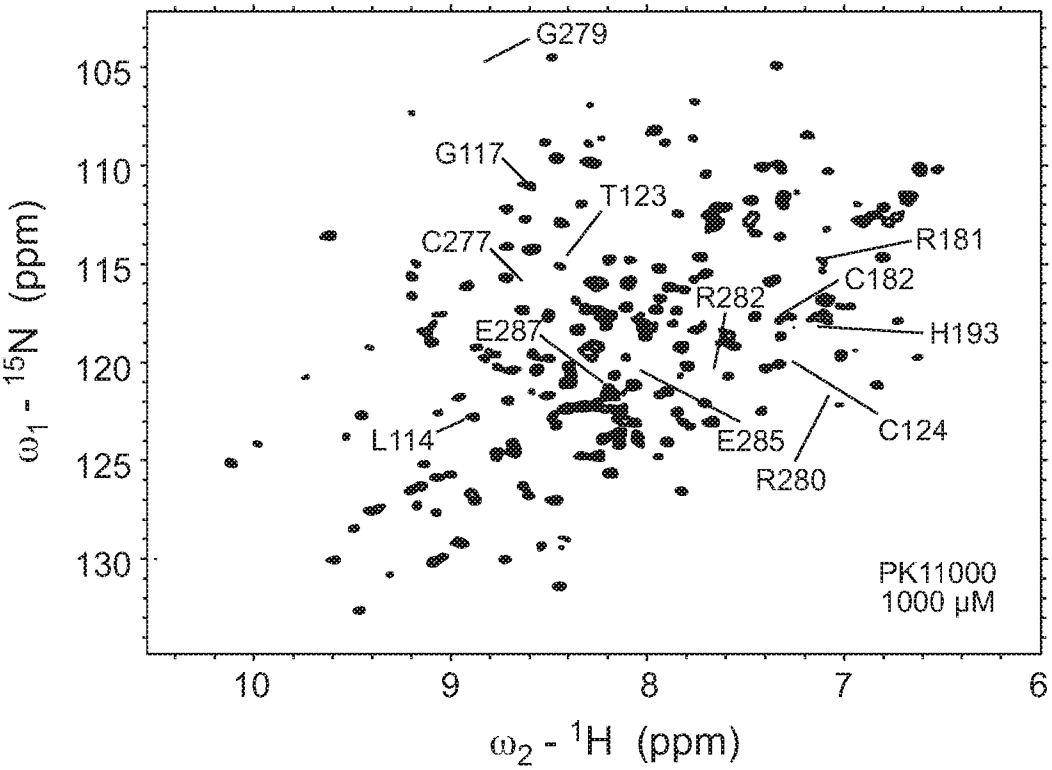


Figure 9

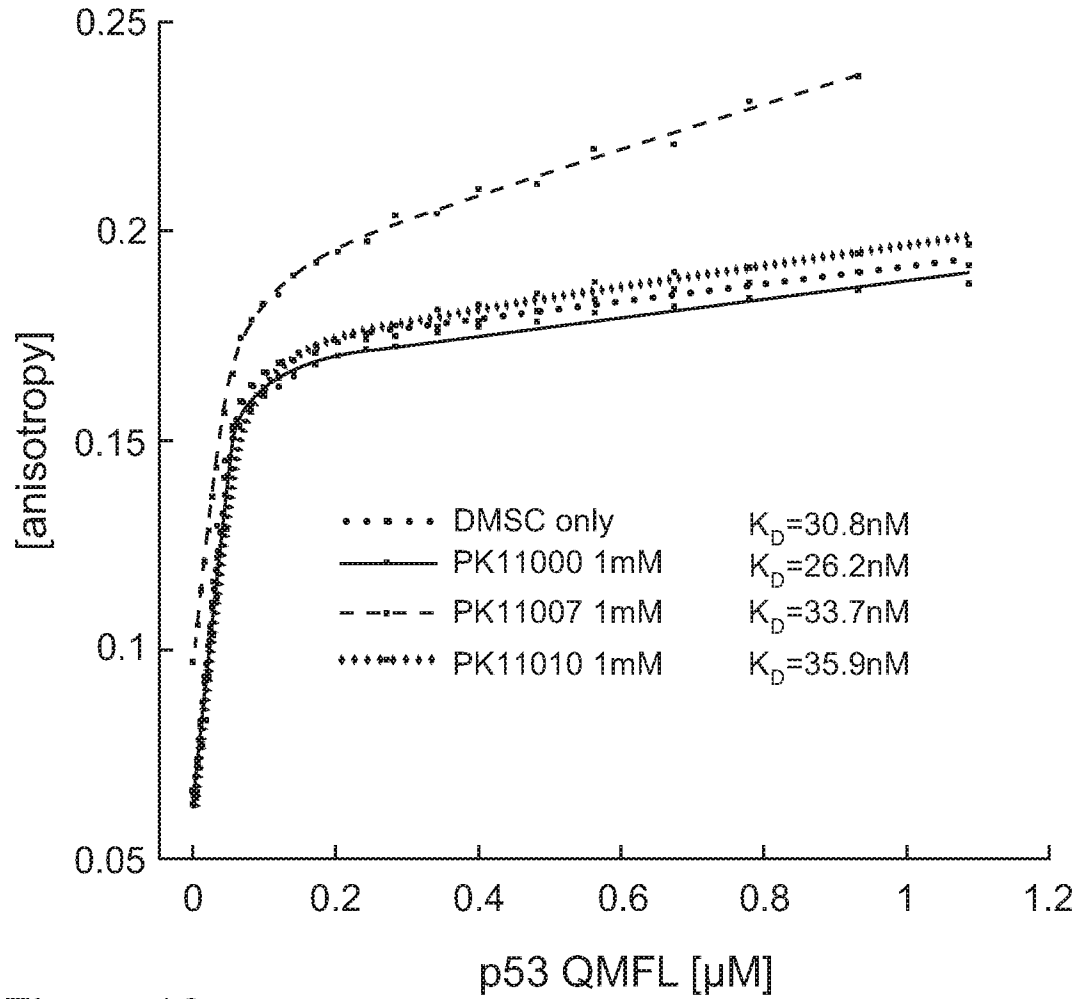


Figure 10

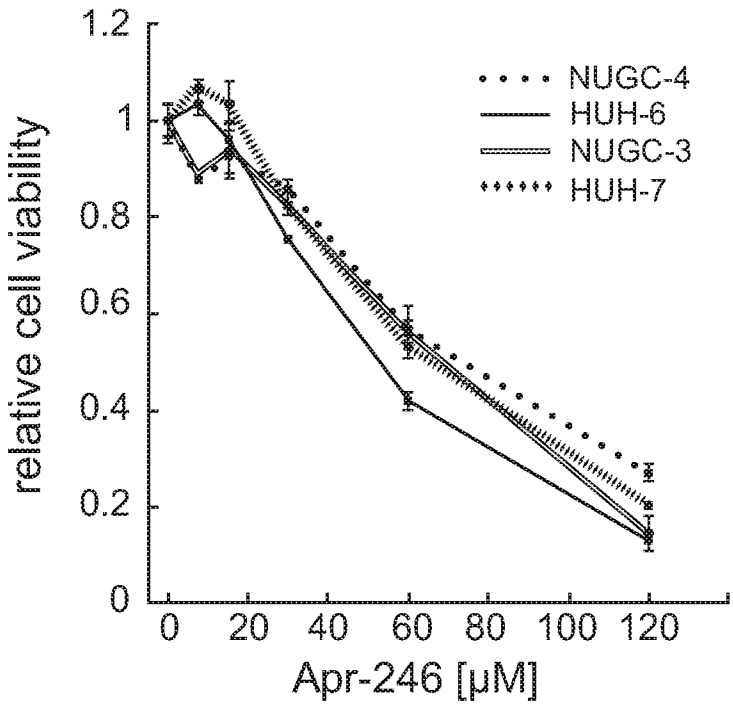


Figure 11A

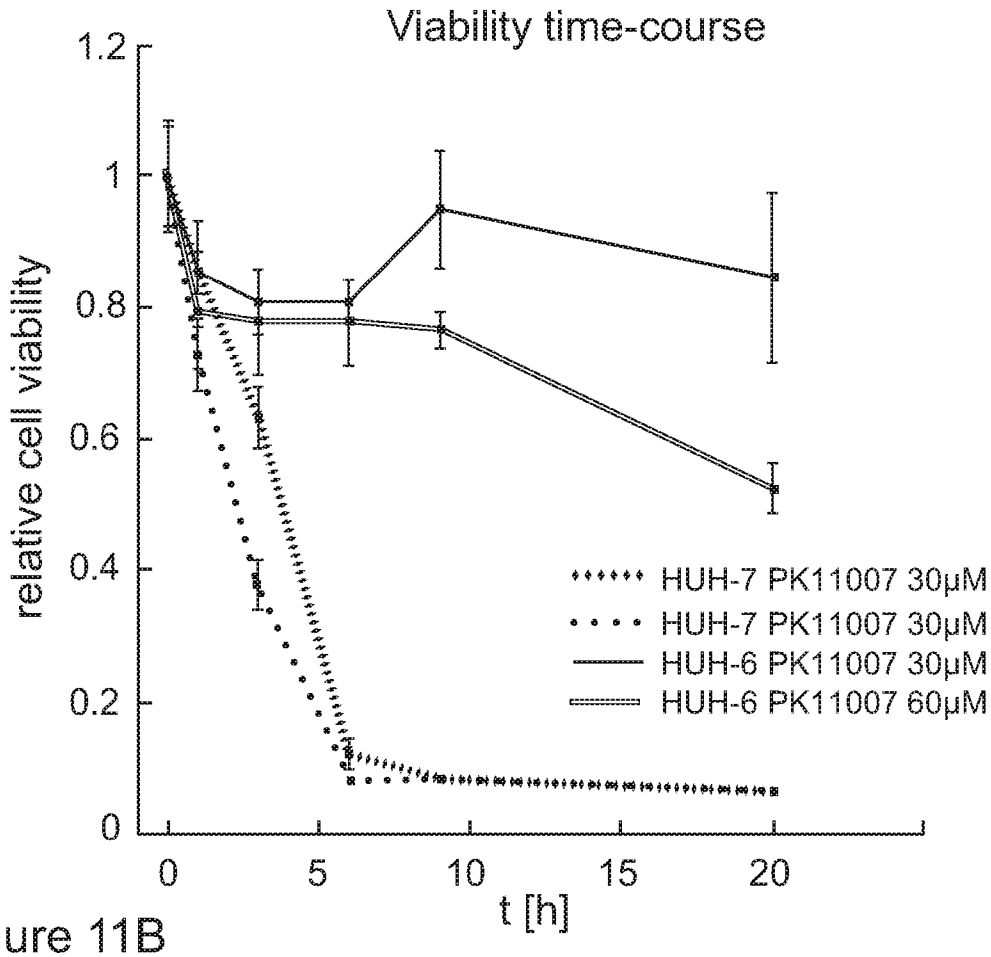


Figure 11B

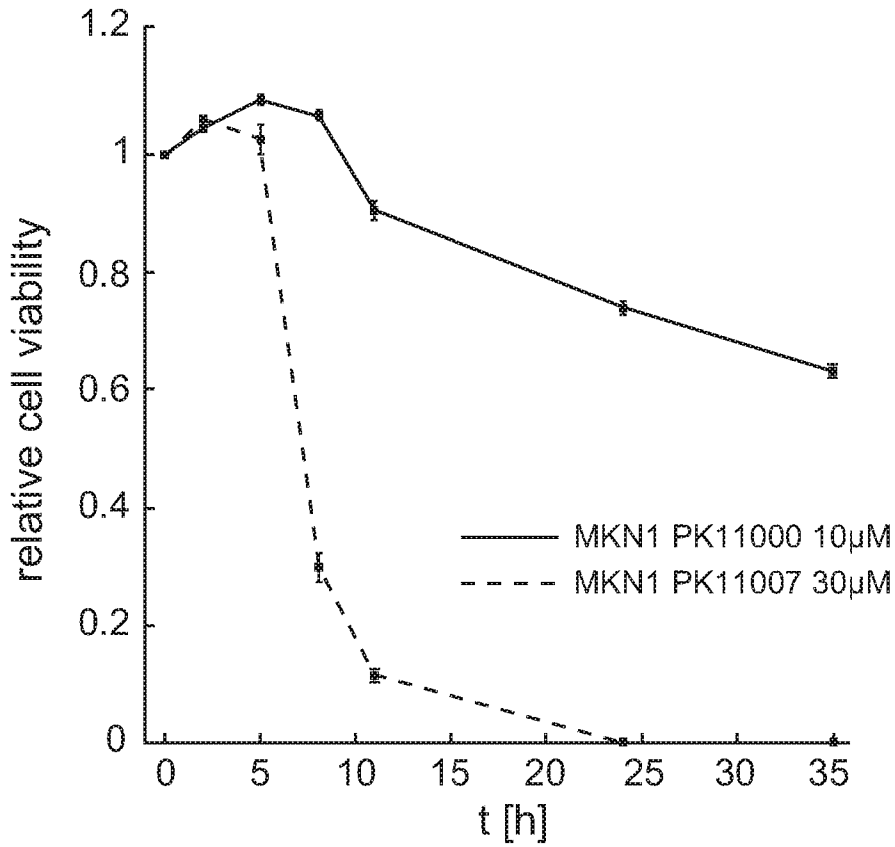


Figure 12

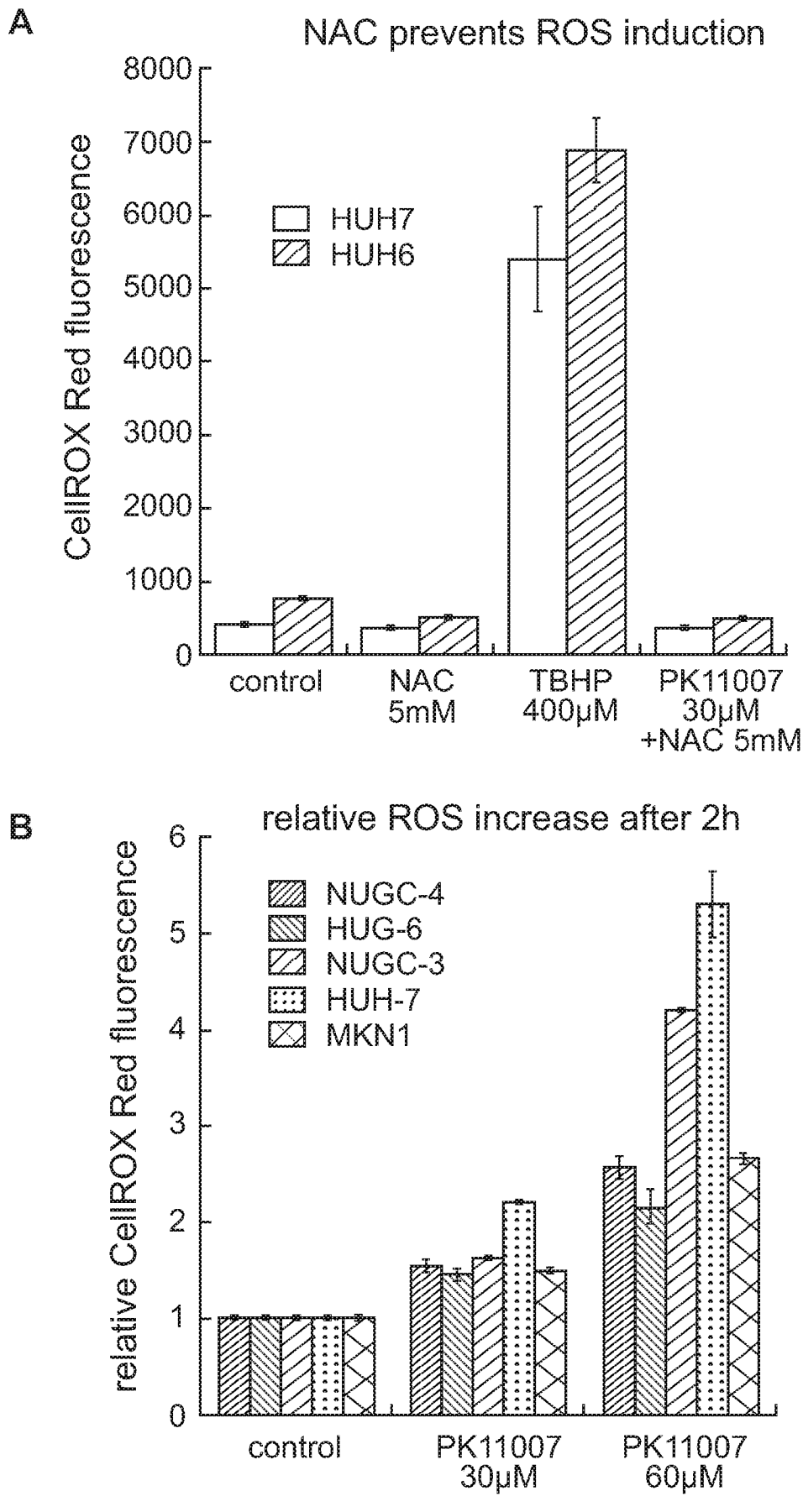
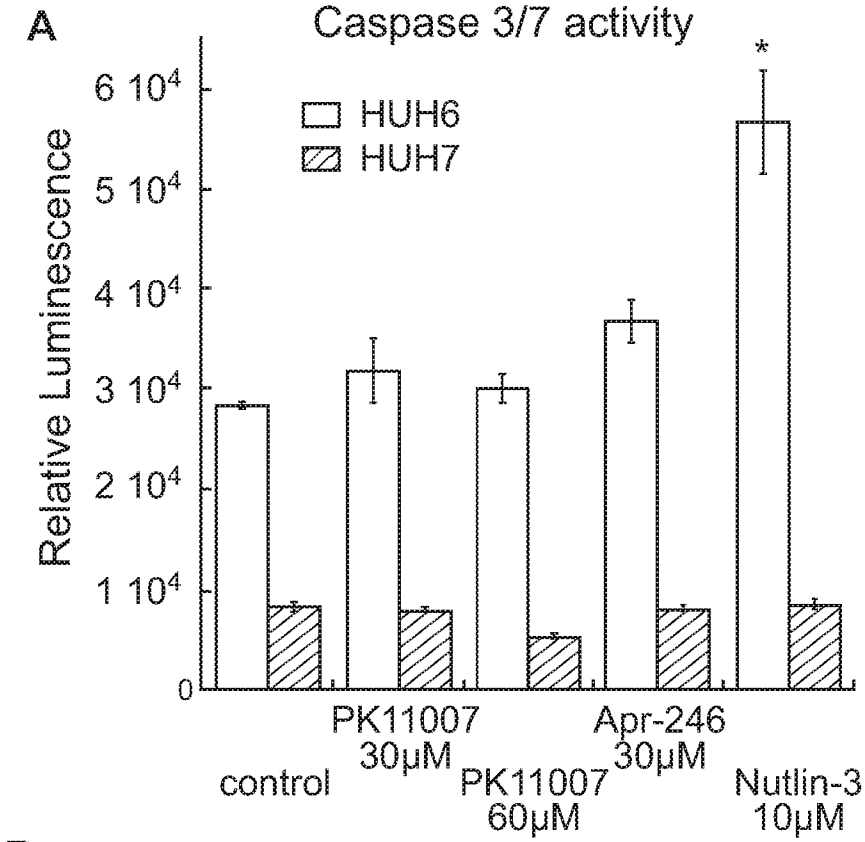

A
NAC prevents ROS induction
CellROX Red fluorescence
8000
7000
6000
5000
4000
3000
2000
1000
0
HUH7
HUH6
control
NAC 5mM
TBHP 400µM
PK11007 30µM +NAC 5mM
B
relative ROS increase after 2h
relative CellROX Red fluorescence
6
5
4
3
2
1
0
NUGC-4
HUG-6
NUGC-3
HUH-7
MKN1
control
PK11007 30µM
PK11007 60µM

Figure 13


A
Caspase 3/7 activity
Relative Luminescence
6 10^4
5 10^4
4 10^4
3 10^4
2 10^4
1 10^4
0
HUH6
HUH7
*
control
PK11007 30μM
PK11007 60μM
Apr-246 30μM
Nutlin-3 10μM

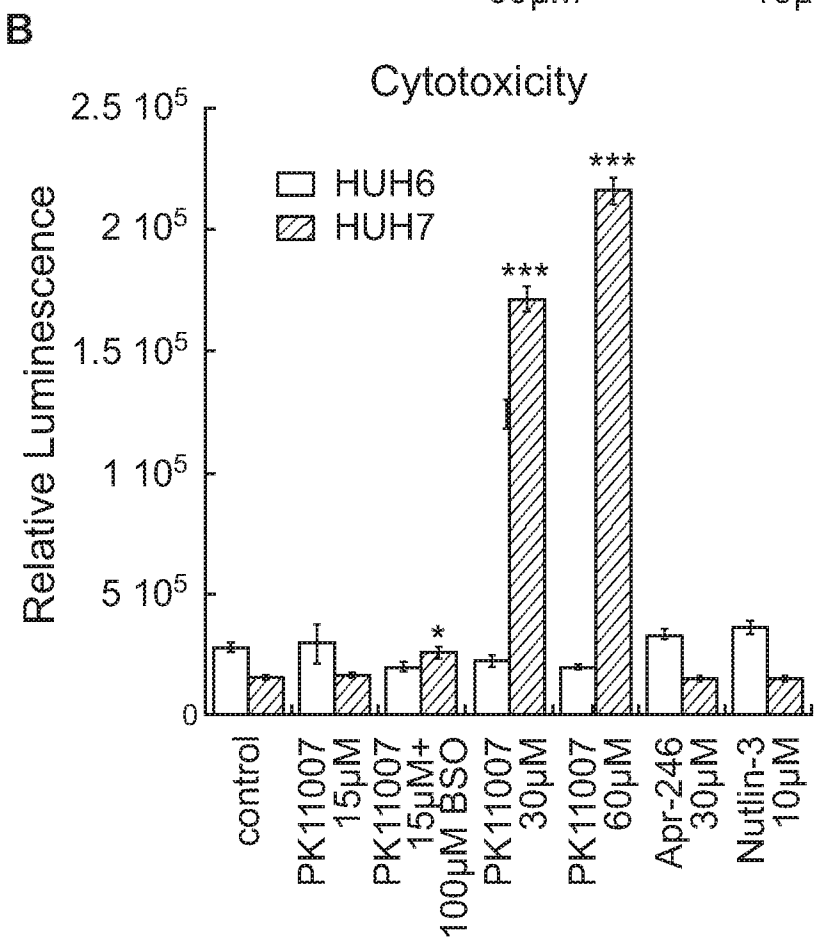

B
Cytotoxicity
Relative Luminescence
2.5 10^5
2 10^5
1.5 10^5
1 10^5
5 10^5
0
HUH6
HUH7
*
***
***
control
PK11007 15μM
PK11007 15μM+ 100μM BSO
PK11007 30μM
PK11007 60μM
Apr-246 30μM
Nutlin-3 10μM

Figure 13 continued

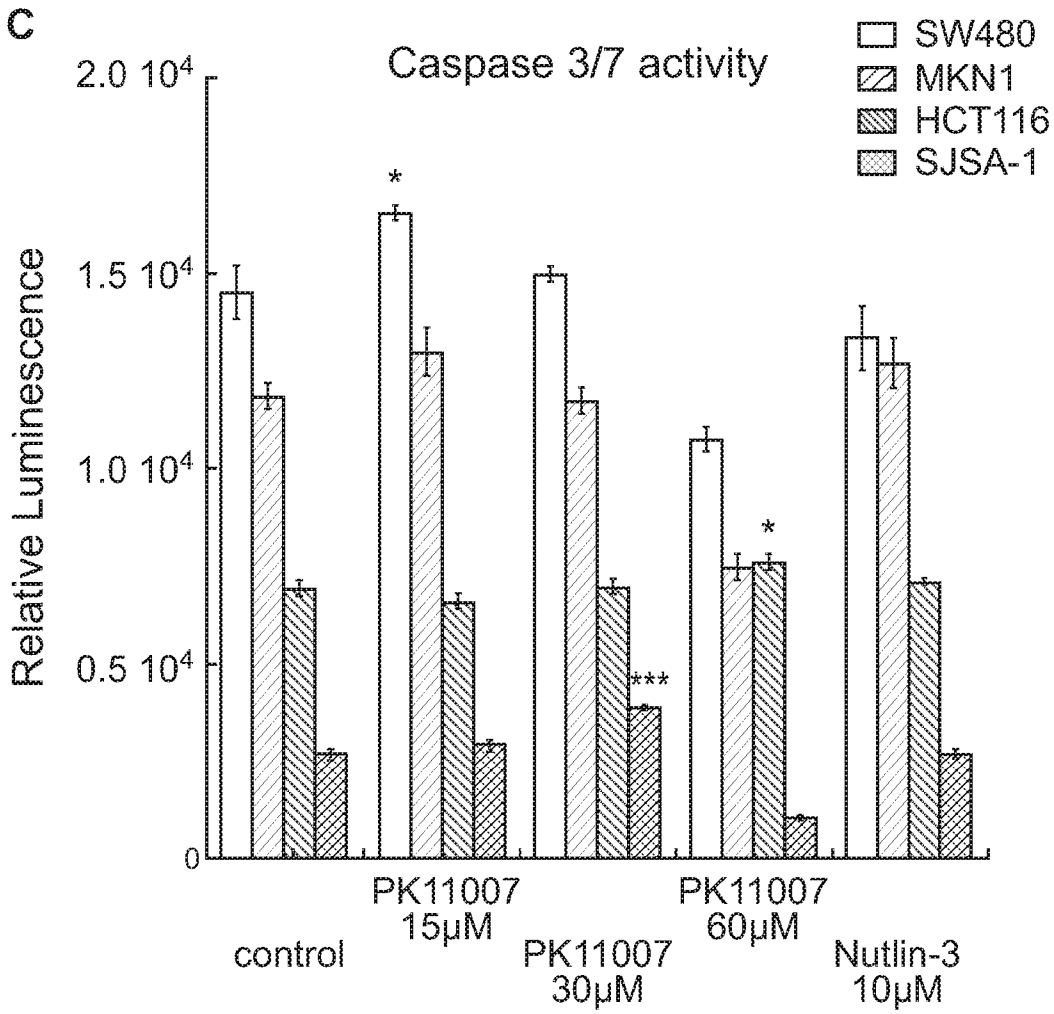


Figure 14

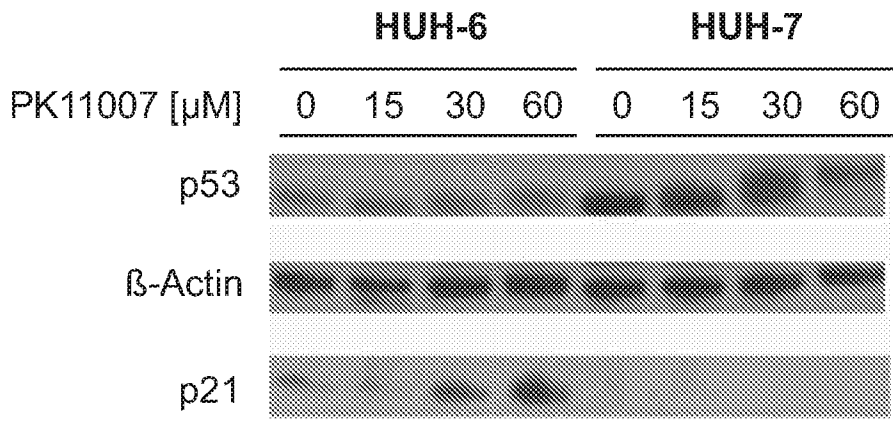


Figure 15A

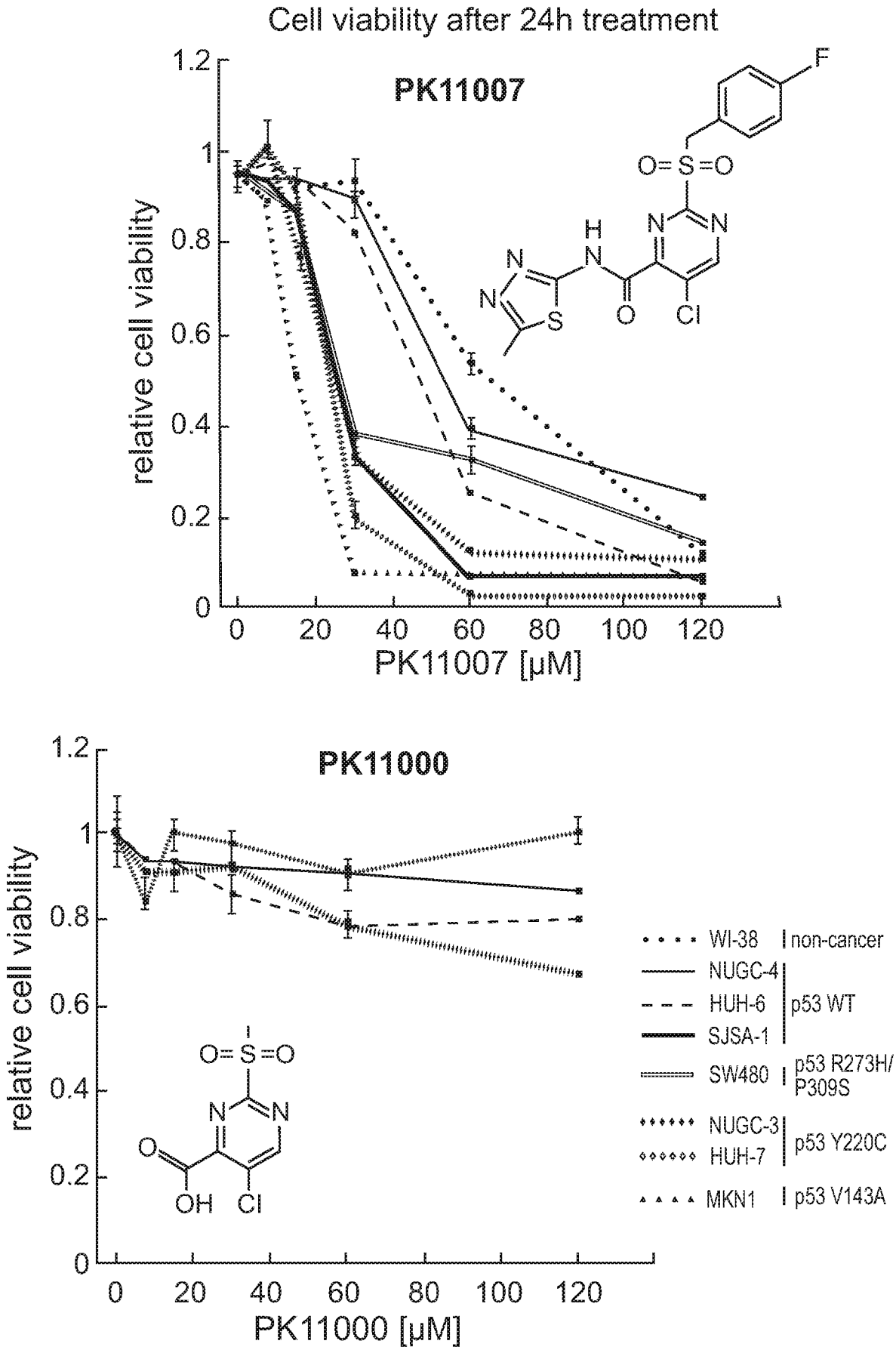

Cell viability after 24h treatment
PK11007
relative cell viability
PK11007 [µM]
PK11000
relative cell viability
PK11000 [µM]
WI-38
NUGC-4
HUH-6
SJSA-1
SW480
NUGC-3
HUH-7
MKN1
non-cancer
p53 WT
p53 R273H/ P309S
p53 Y220C
p53 V143A

Figure 15A continued

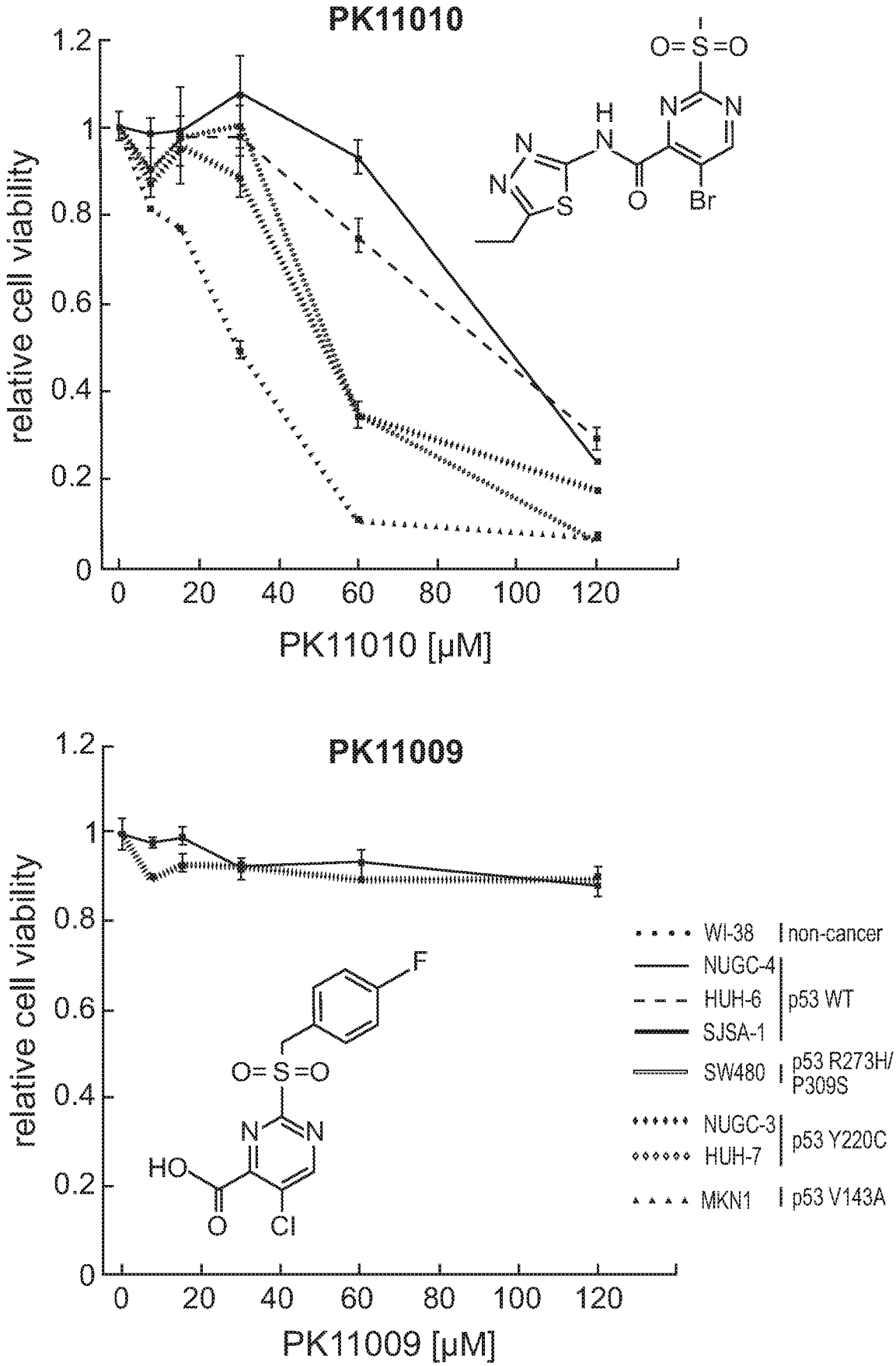


Figure 15A continued

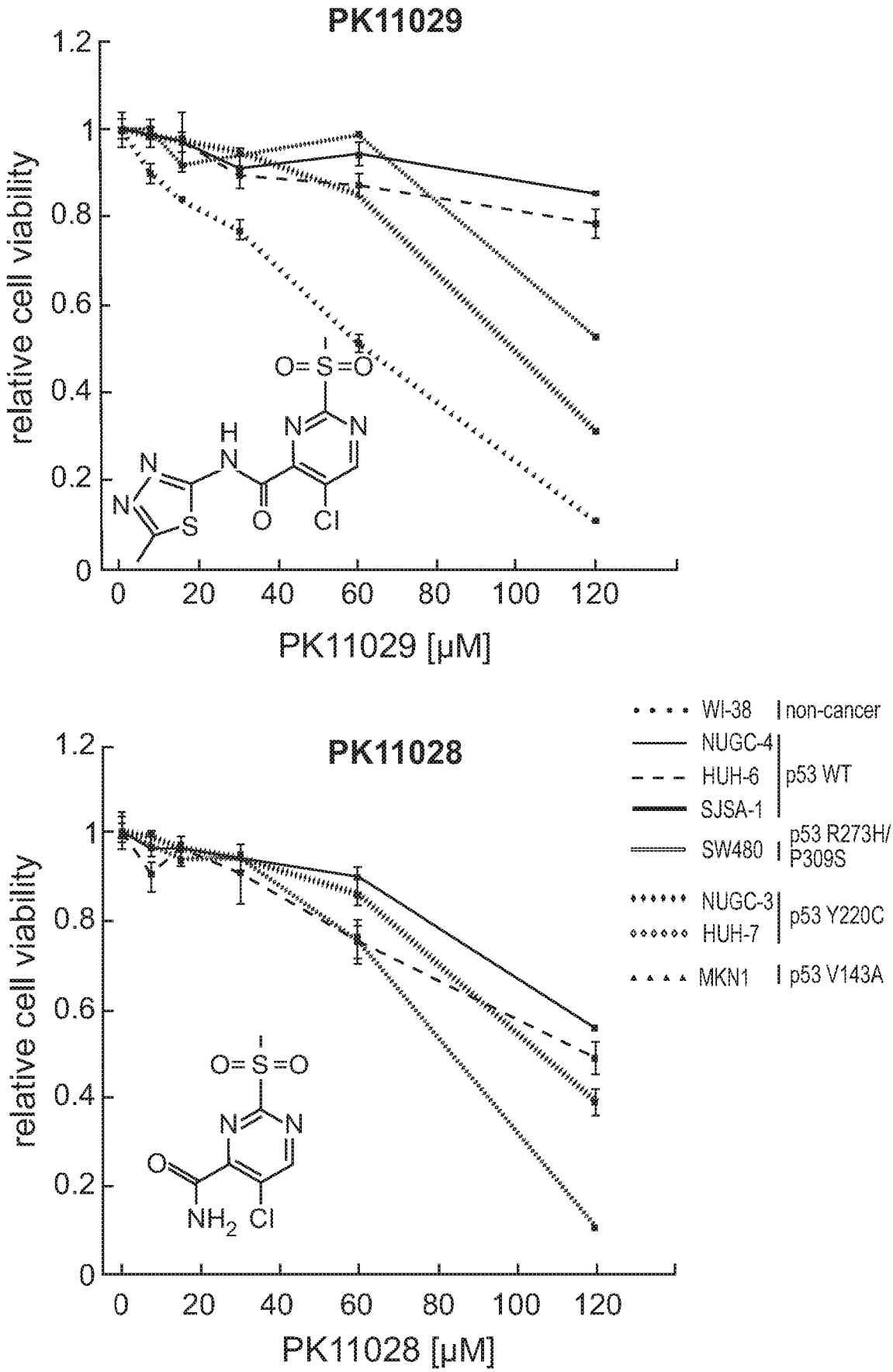
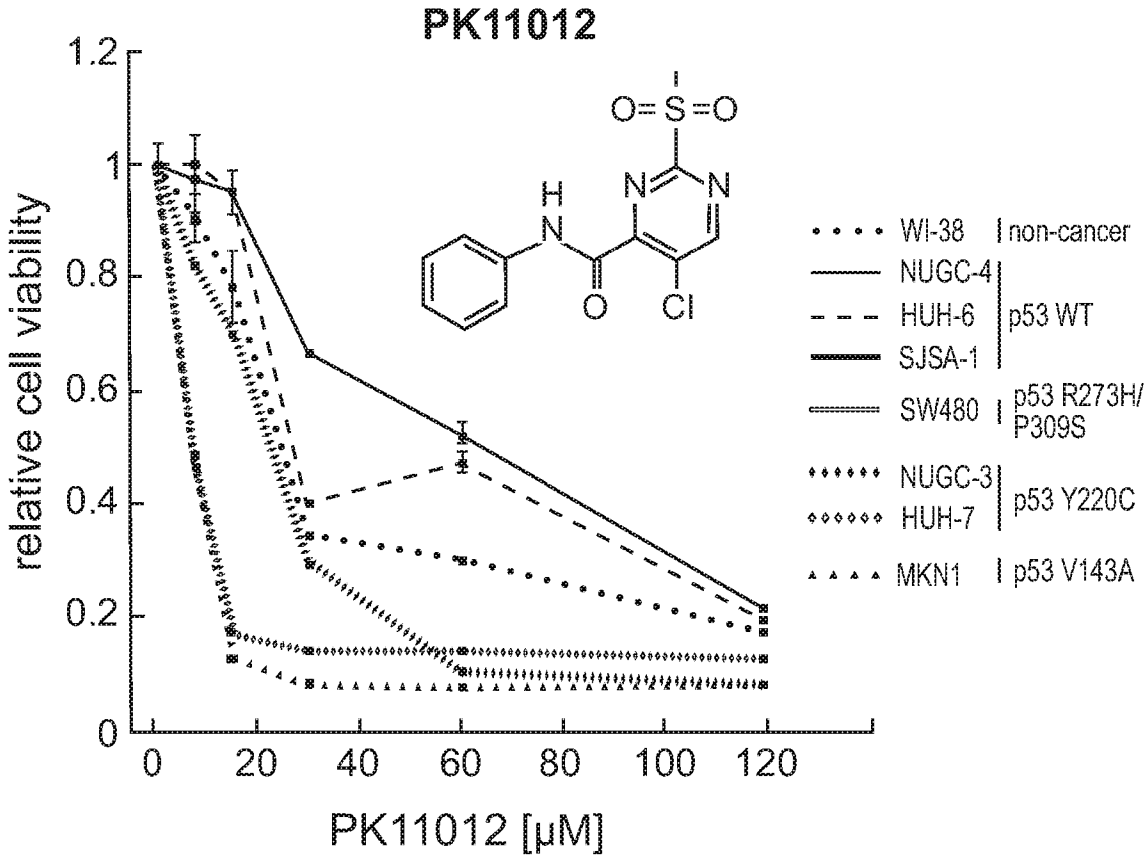


Figure 15B


PK11012
relative cell viability
1.2
1
0.8
0.6
0.4
0.2
0
0 20 40 60 80 100 120
PK11012 [μM]
O= S =O
H
N
N
N
O
Cl
WI-38 non-cancer
NUGC-4
HUH-6 p53 WT
SJSA-1
SW480 p53 R273H/ P309S
NUGC-3
HUH-7 p53 Y220C
MKN1 p53 V143A

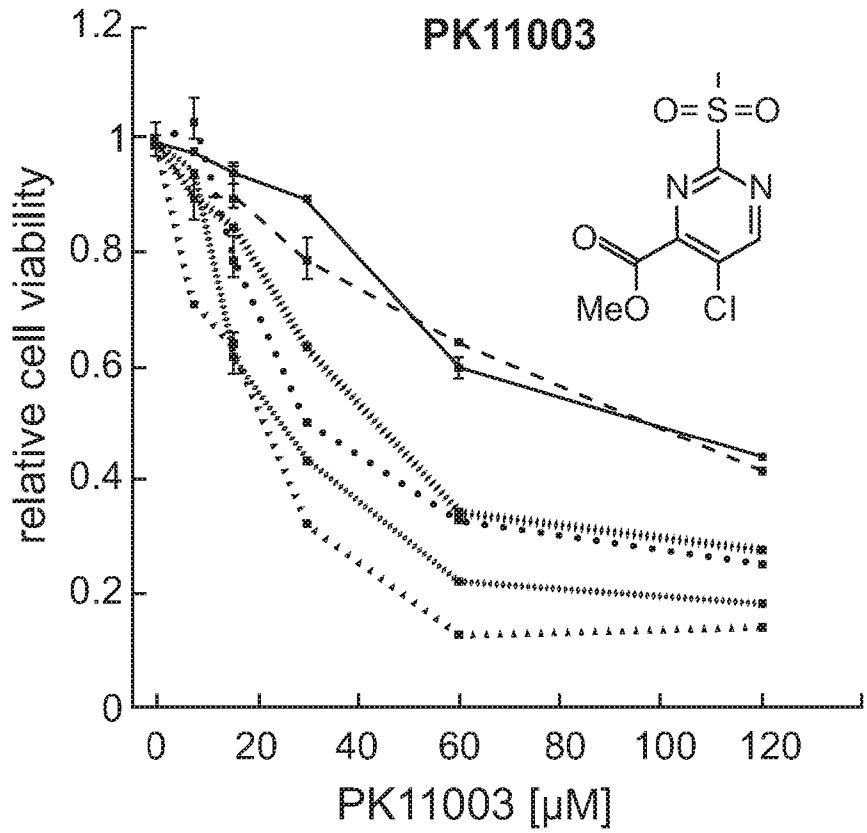

PK11003
relative cell viability
1.2
1
0.8
0.6
0.4
0.2
0
0 20 40 60 80 100 120
PK11003 [μM]
O= S =O
N
N
O
MeO
Cl

Figure 15B continued

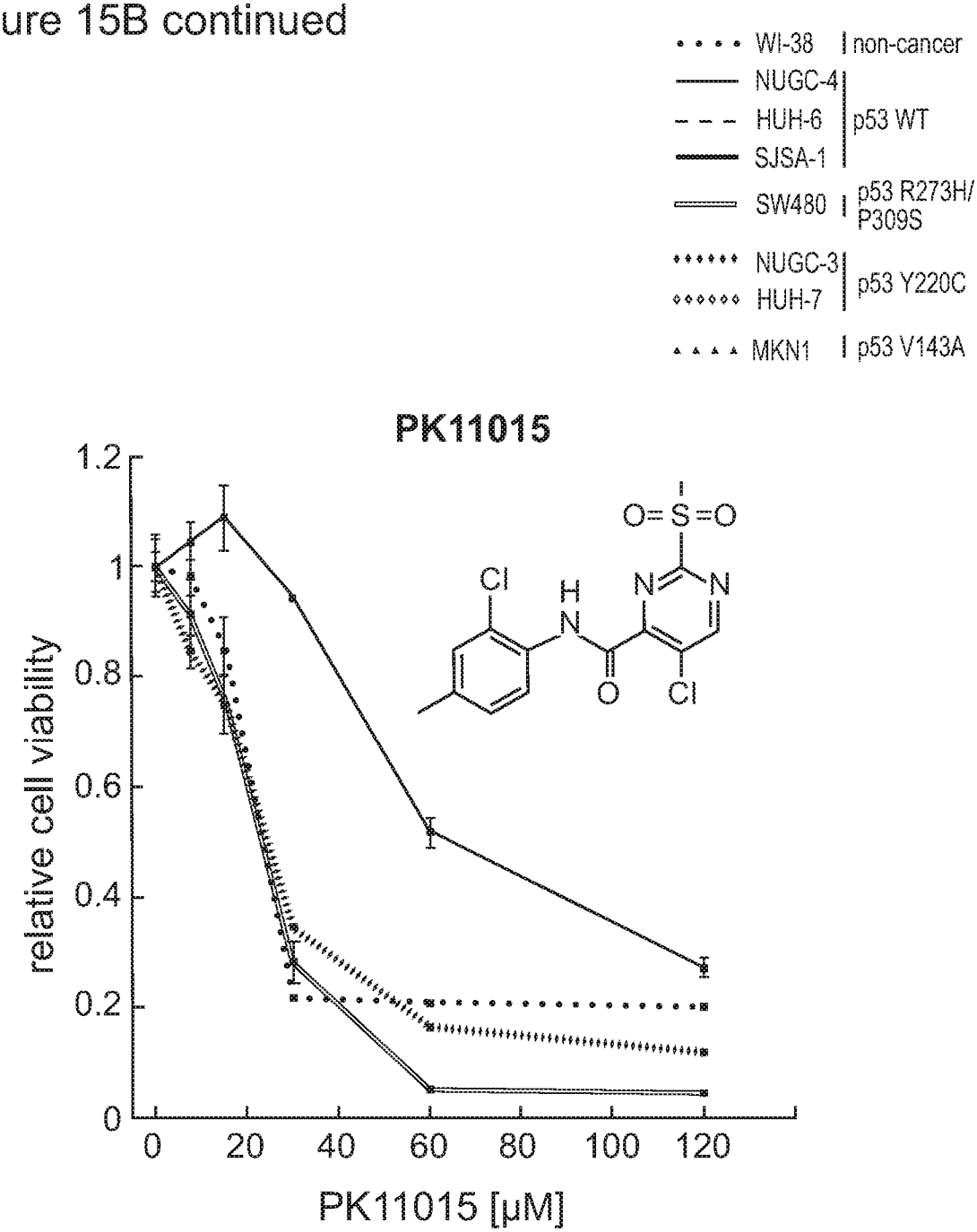


Figure 16

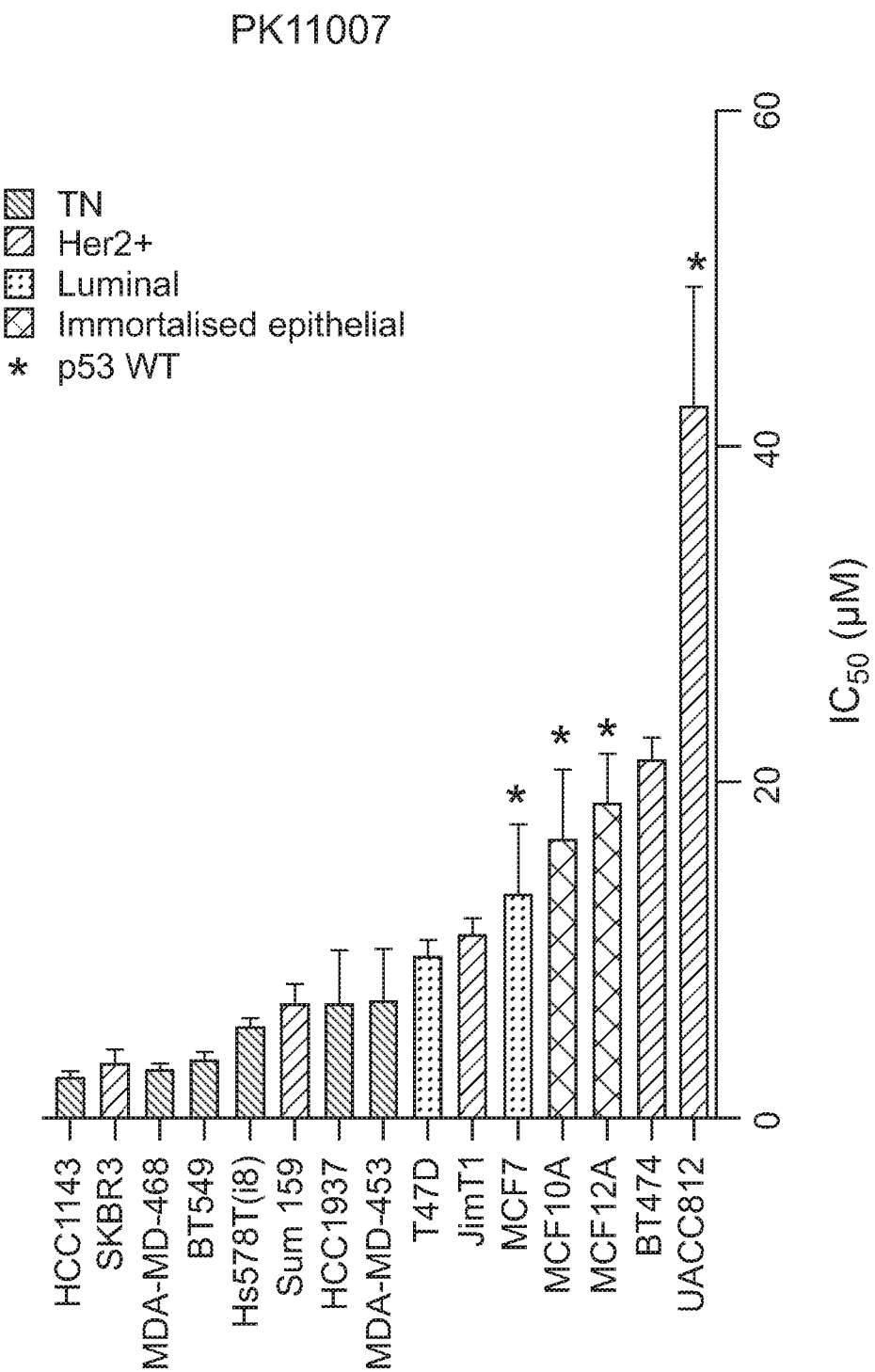
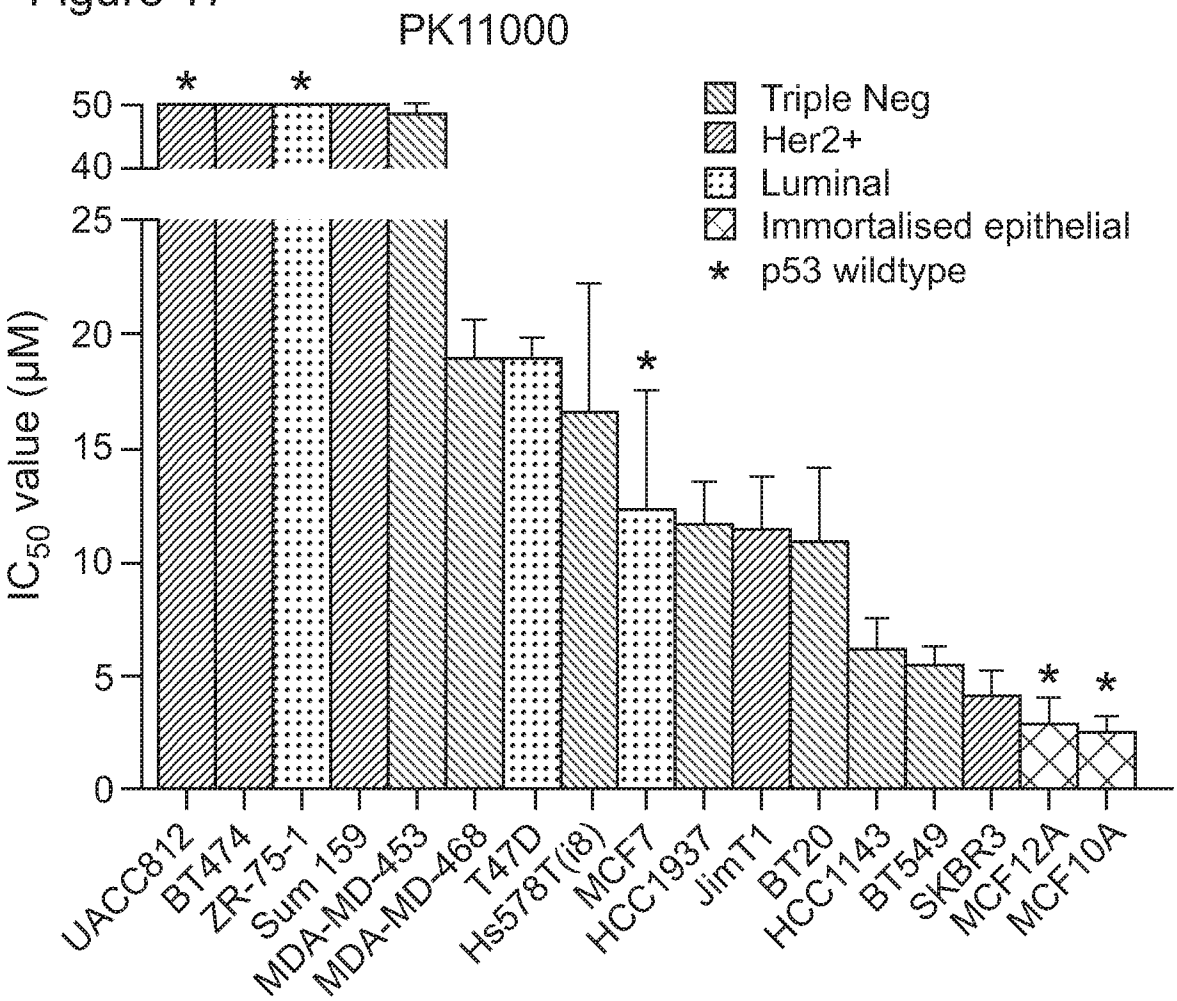
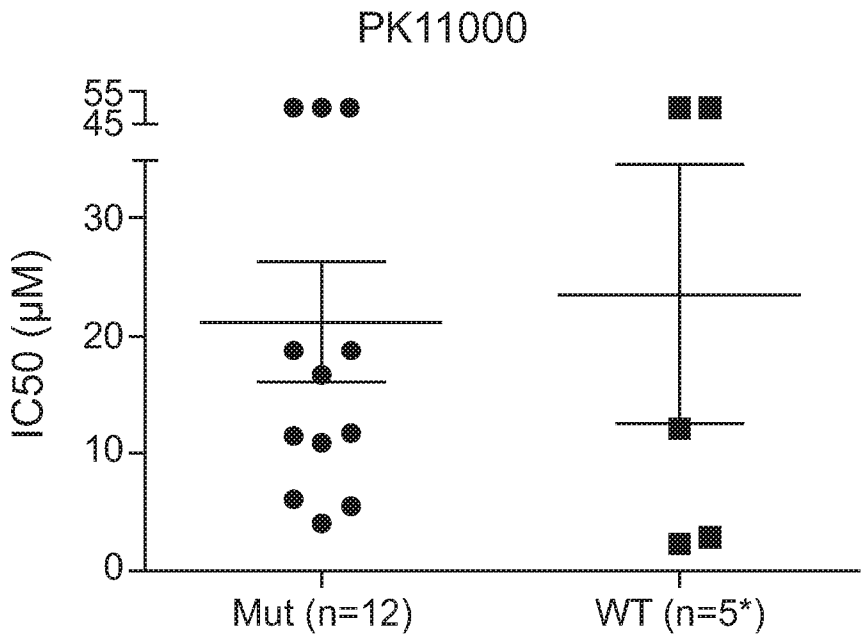

PK11007
TN
Her2+
Luminal
Immortalised epithelial
* p53 WT
IC50 (µM)
0
20
40
60
HCC1143
SKBR3
MDA-MD-468
BT549
Hs578T(i8)
Sum 159
HCC1937
MDA-MD-453
T47D
JimT1
MCF7
MCF10A
MCF12A
BT474
UACC812

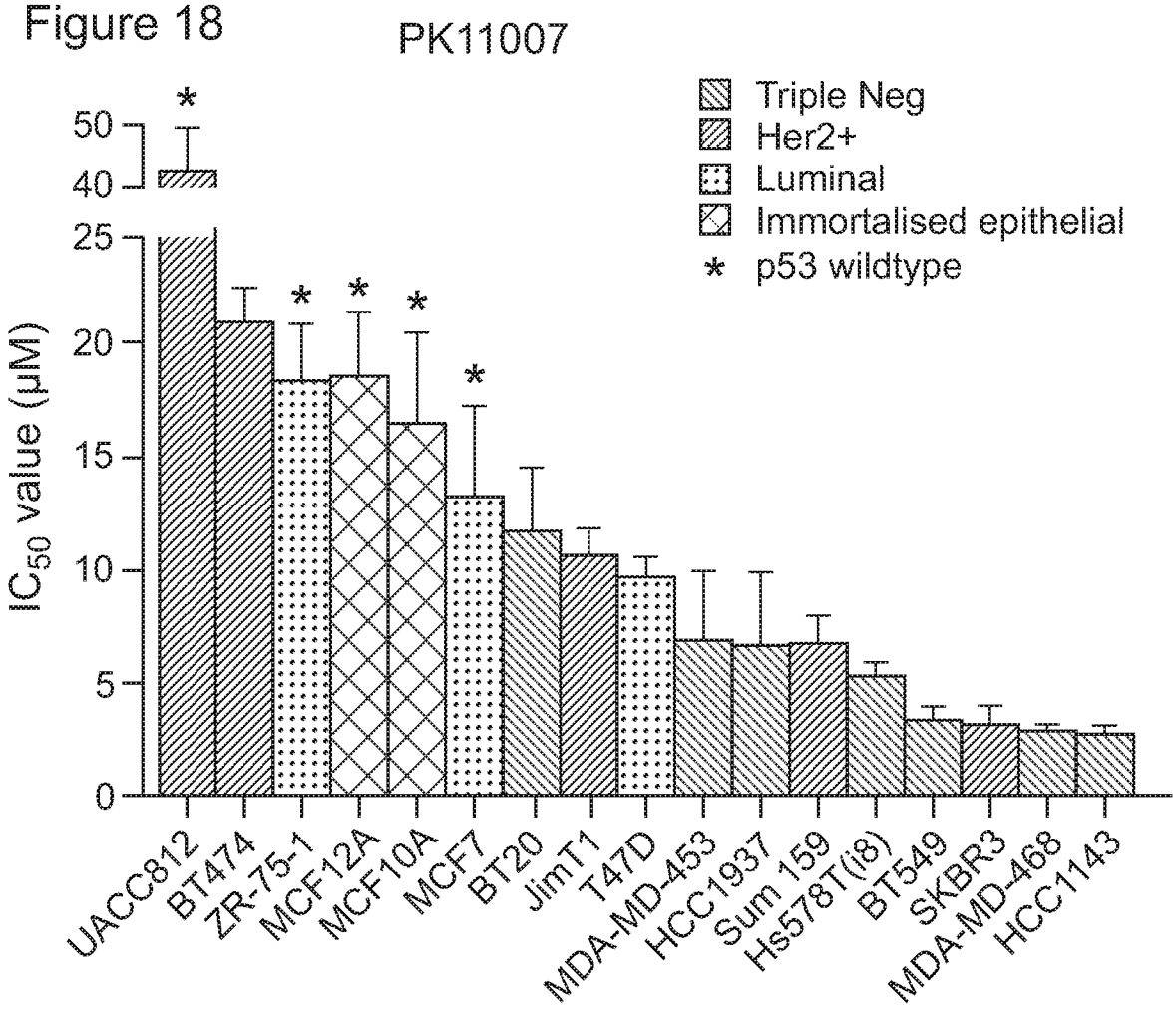
Figure 17

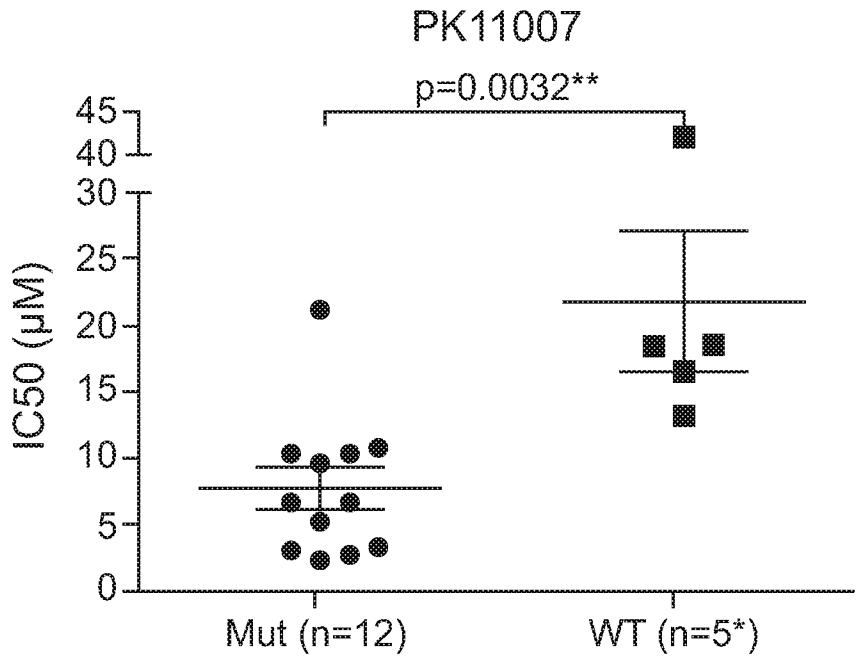




**Two of the p53 WT cells lines used are immortalised breast epithelial cells.*

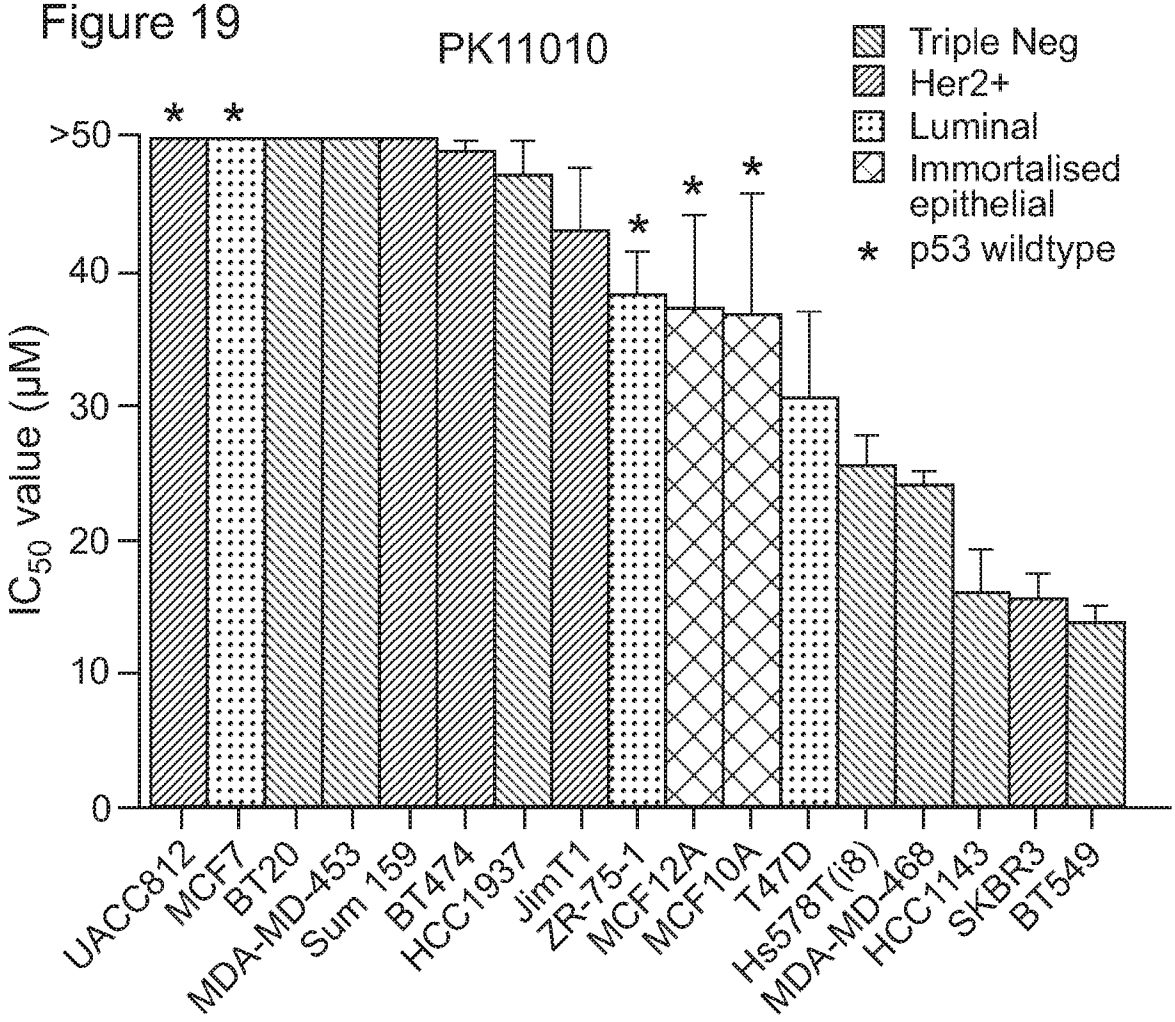
Figure 18

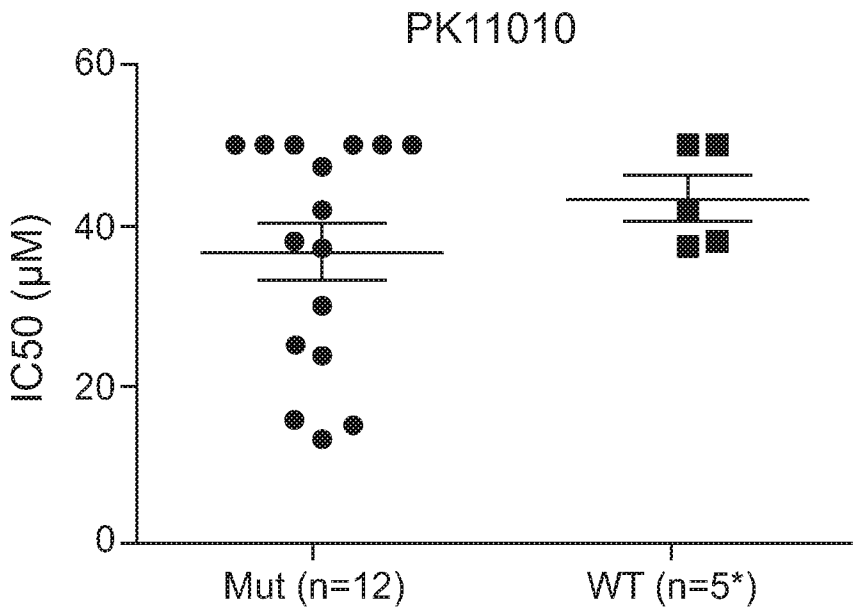




Two of the p53 WT cells lines used are immortalised breast epithelial cells.

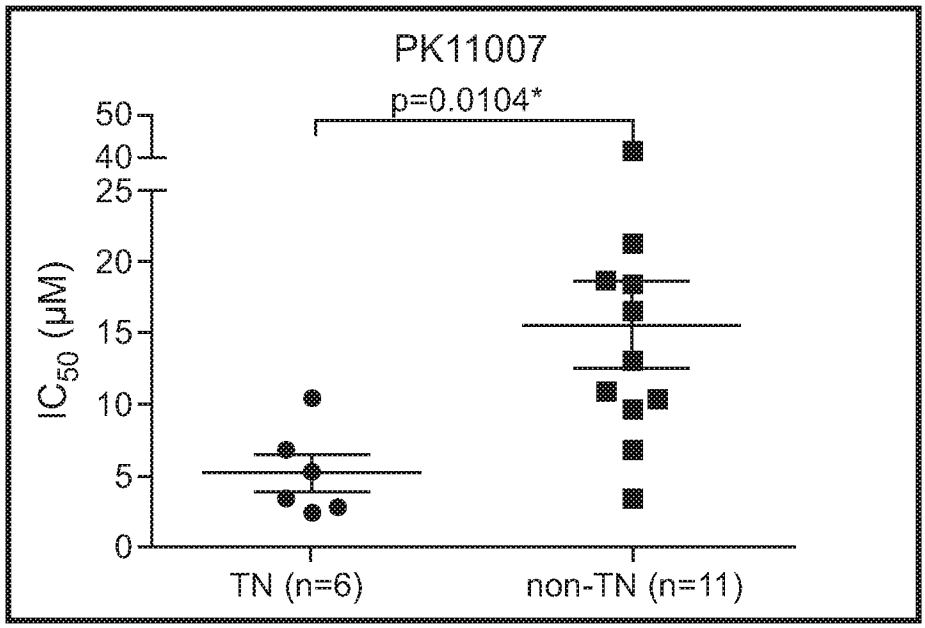
Figure 19

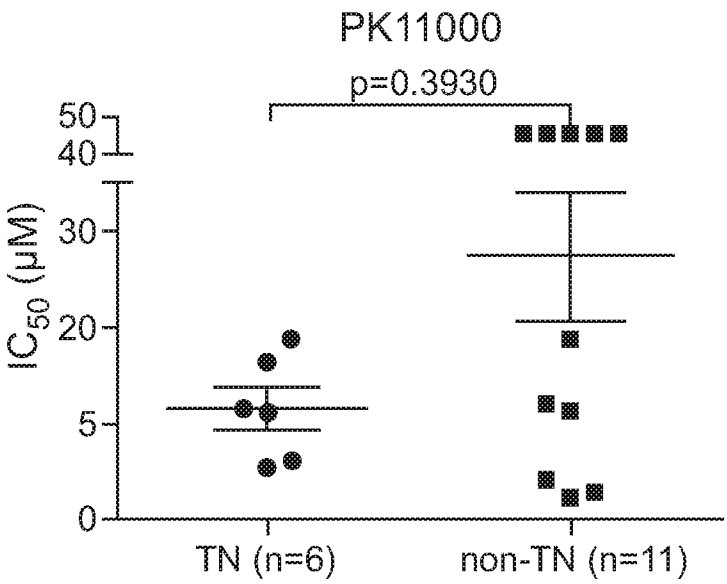


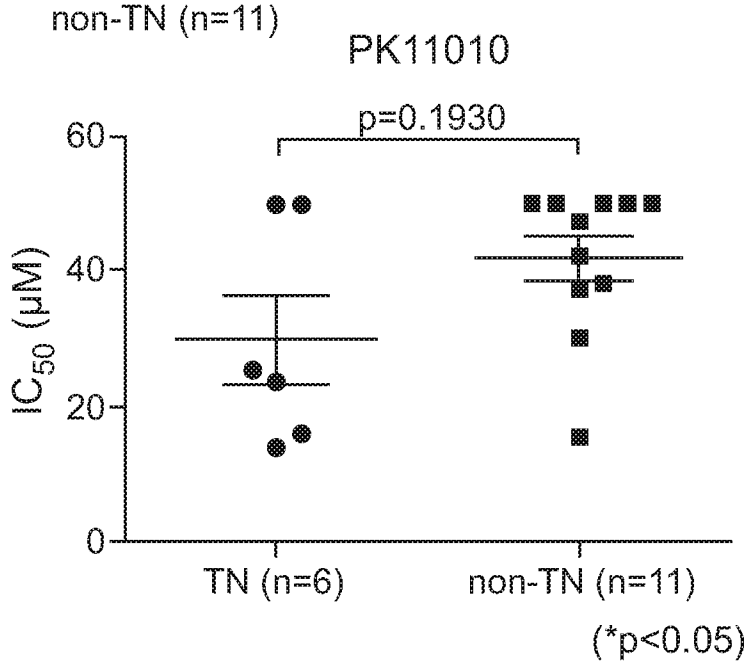


**Two of the p53 WT cells lines used are immortalised breast epithelial cells.* *(*p<0.05)*

Figure 20

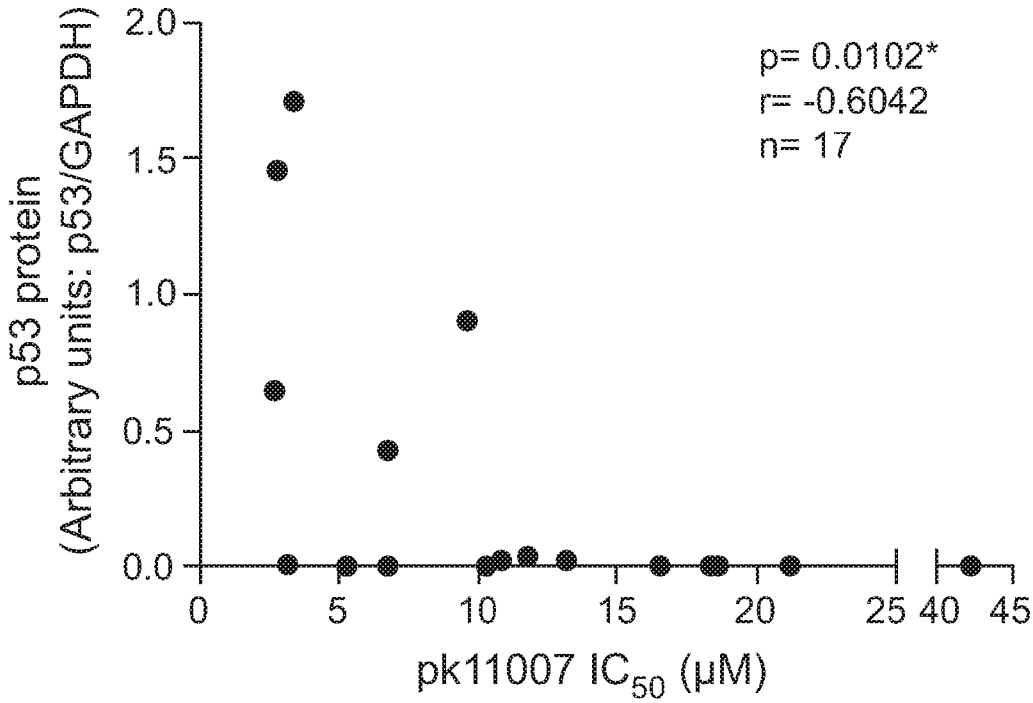


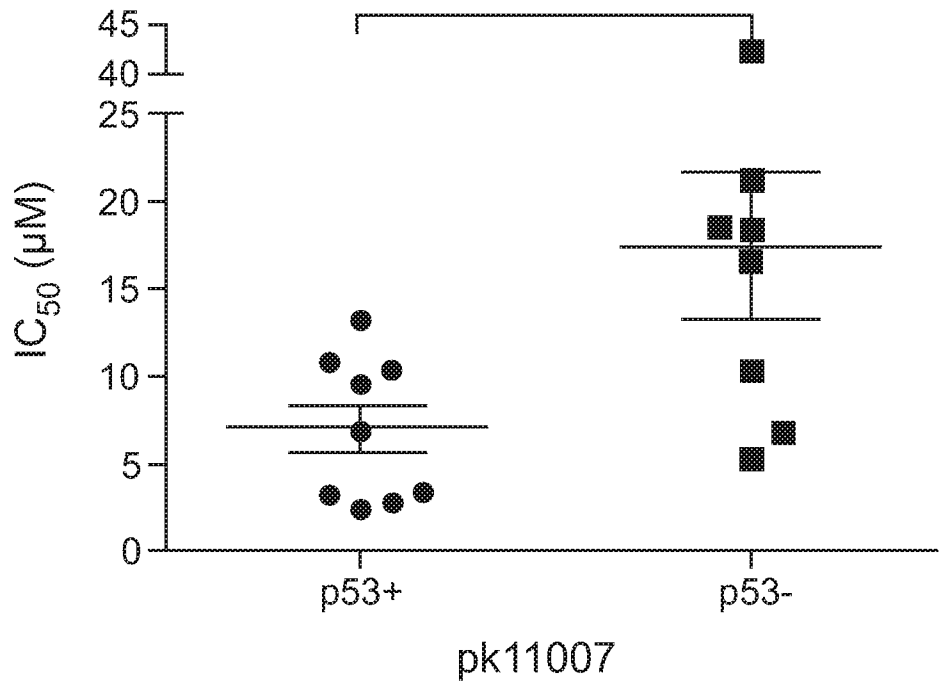




(*p<0.05)

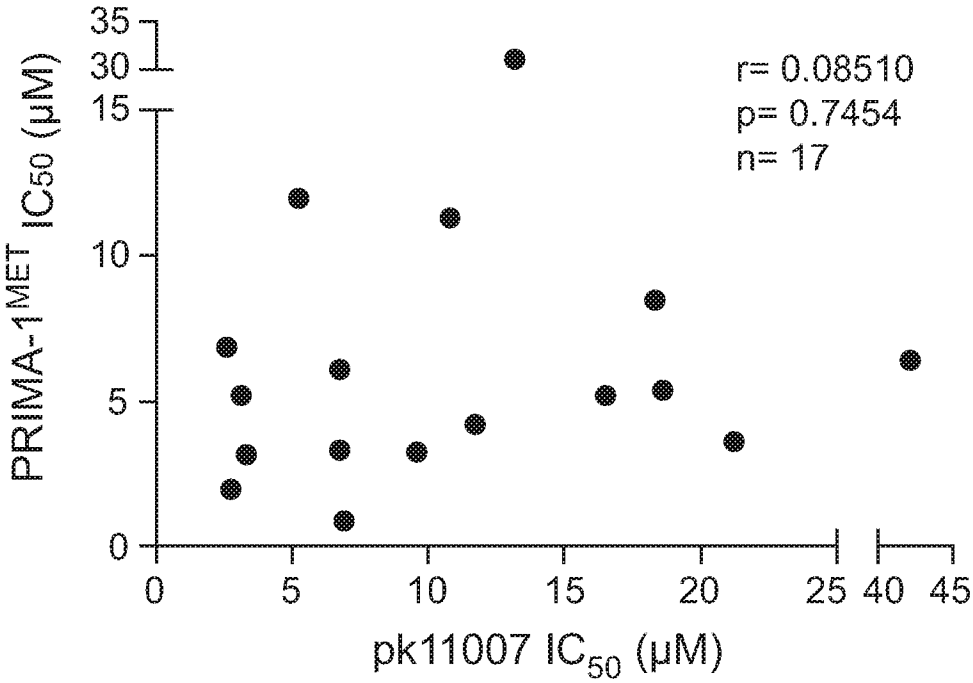
Figure 21

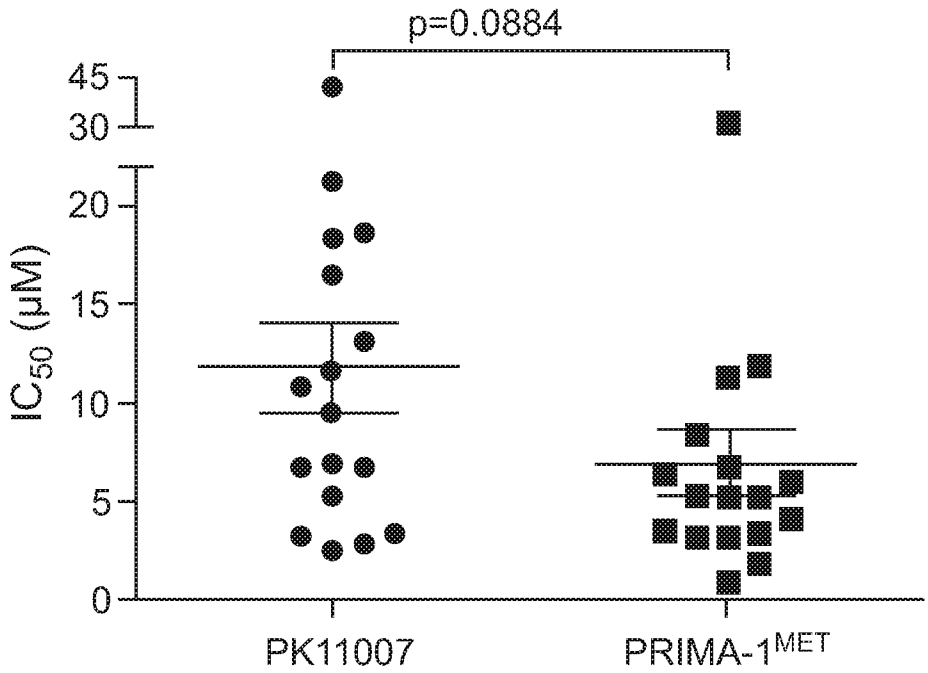




(*p<0.05)

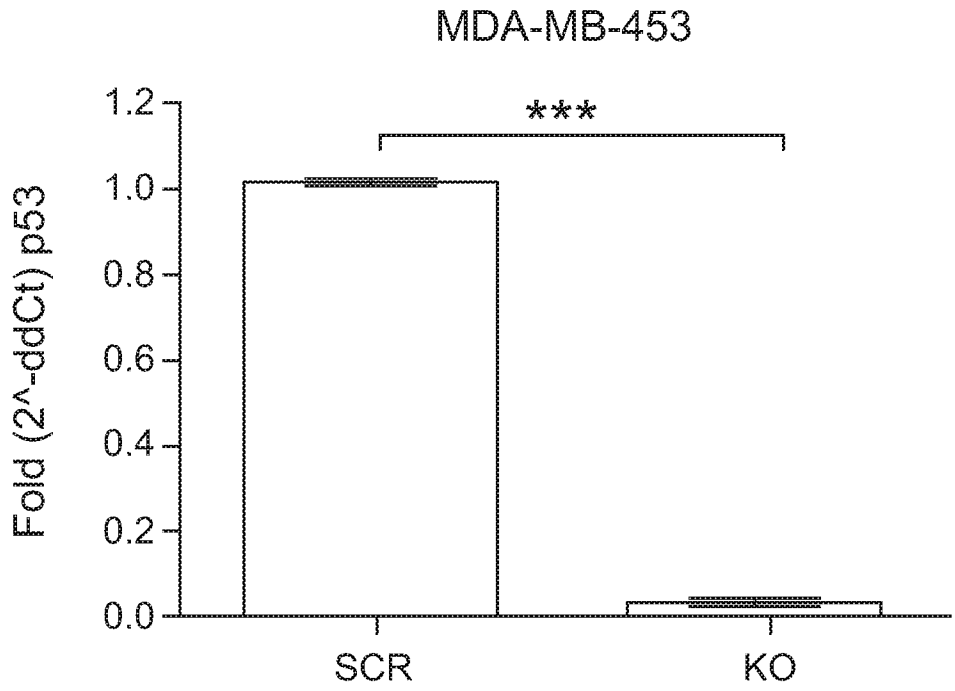
Figure 22





*(*p<0.05)*

Figure 23


MDA-MB-453
***
Fold (2^-ddCt) p53
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SCR
KO

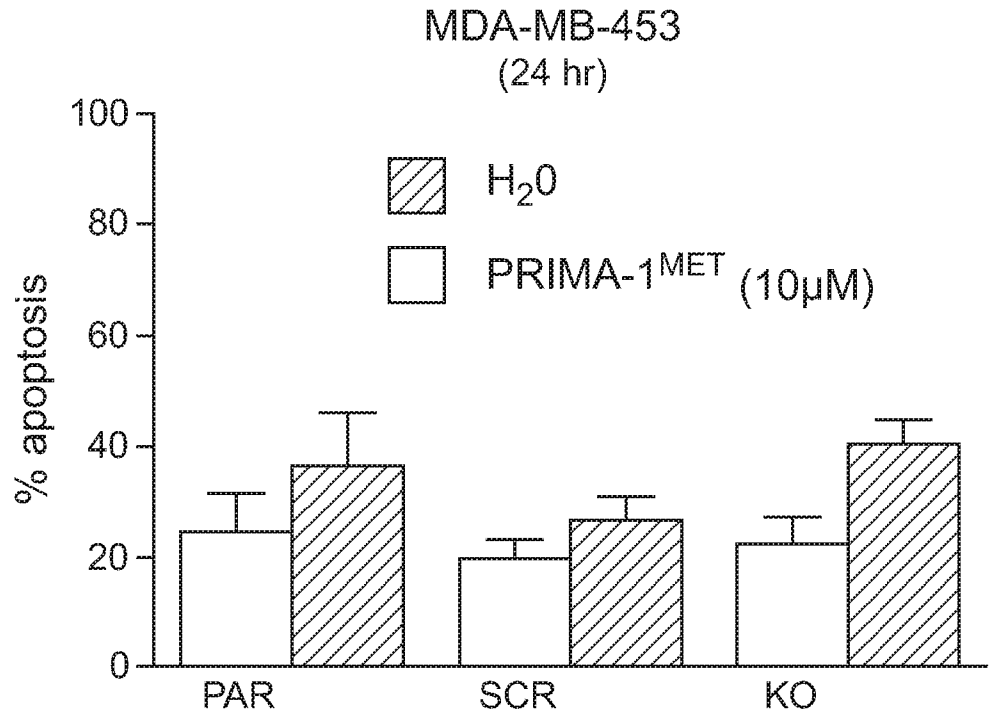

MDA-MB-453
(24 hr)
H20
PRIMA-1MET (10μM)
% apoptosis
100
80
60
40
20
0
PAR
SCR
KO

Figure 24

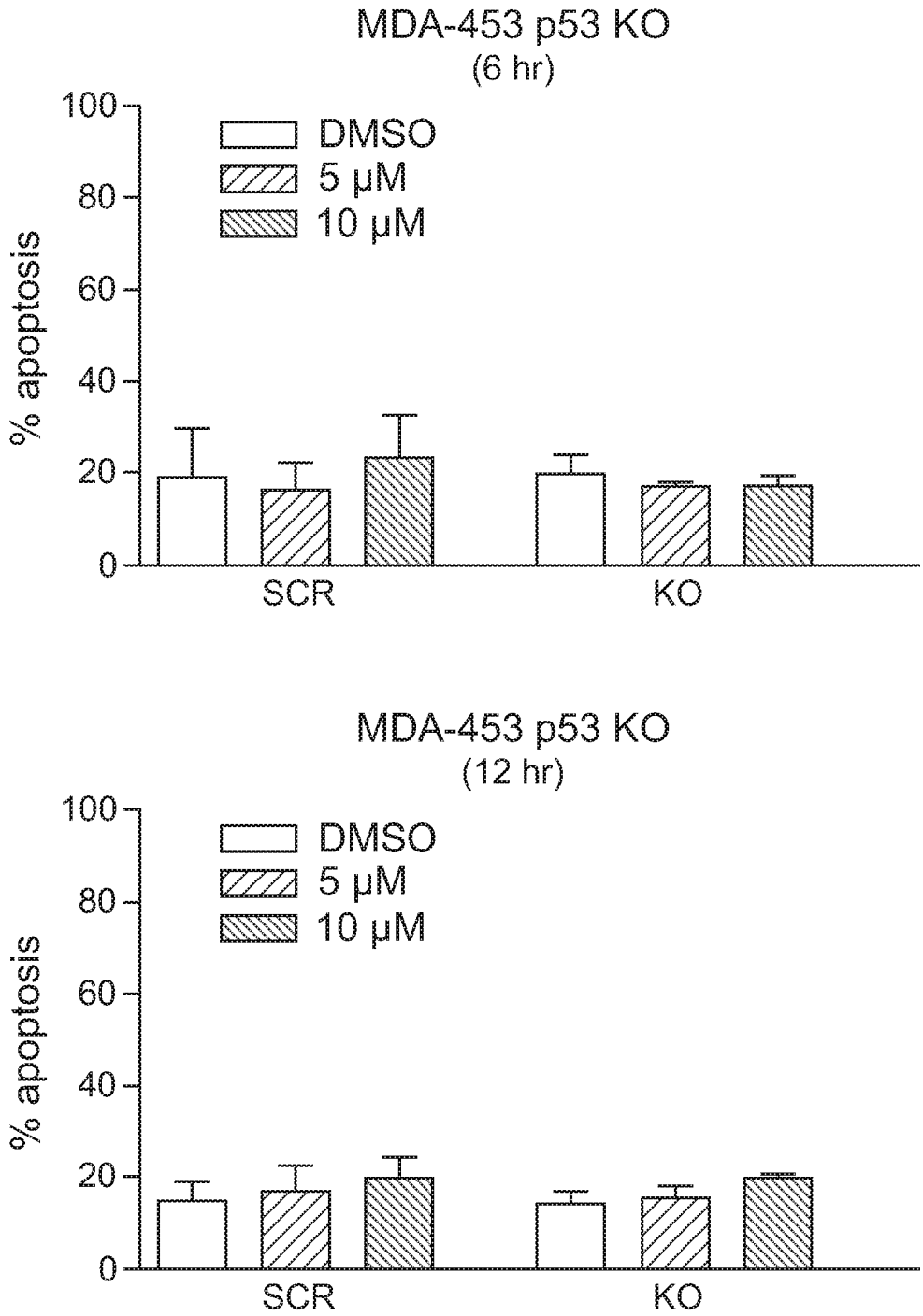

MDA-453 p53 KO
(6 hr)
% apoptosis
100
80
60
40
20
0
DMSO
5 μM
10 μM
SCR
KO
MDA-453 p53 KO
(12 hr)
% apoptosis
100
80
60
40
20
0
DMSO
5 μM
10 μM
SCR
KO

Figure 24 continued

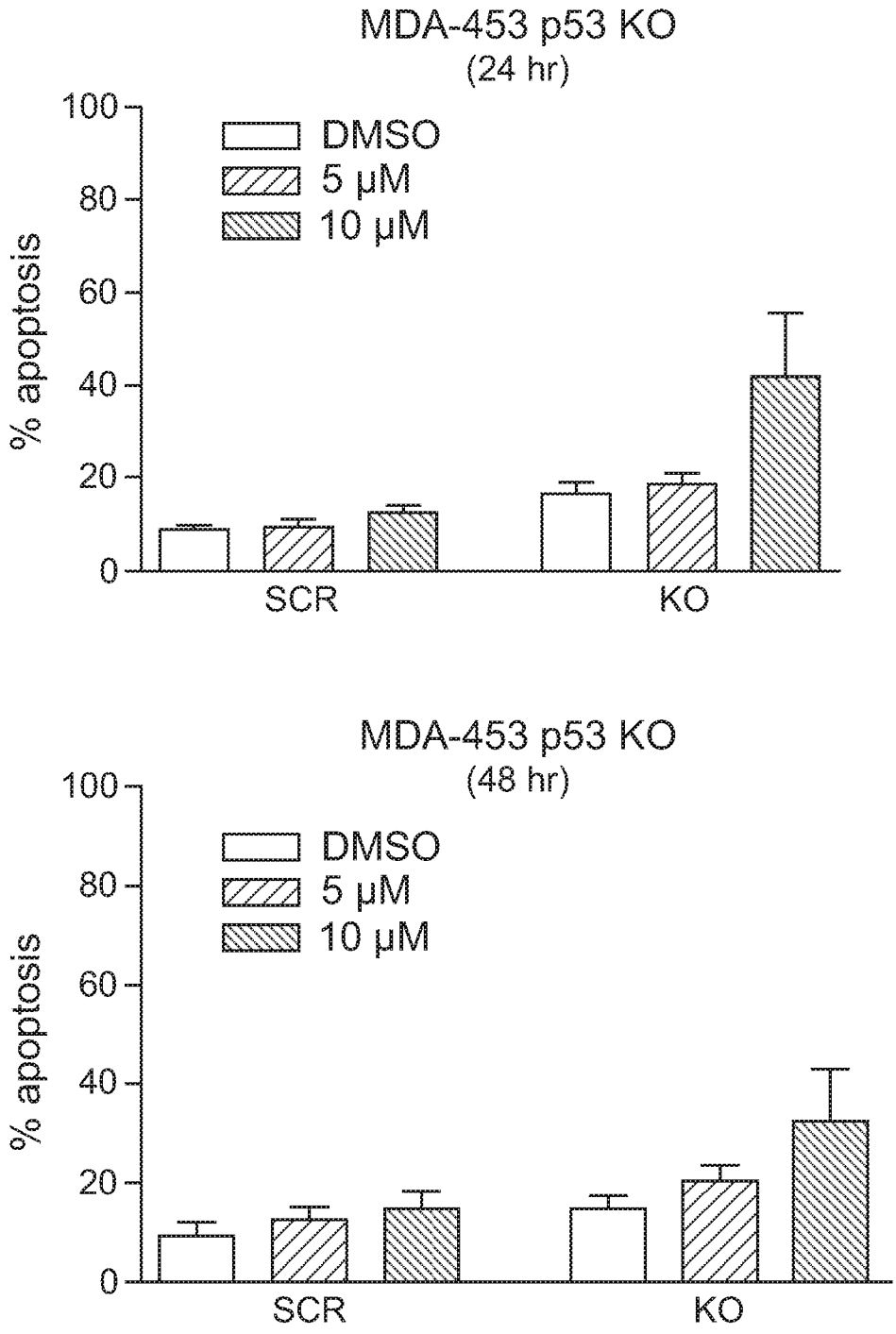


Figure 25

p53 knockdown

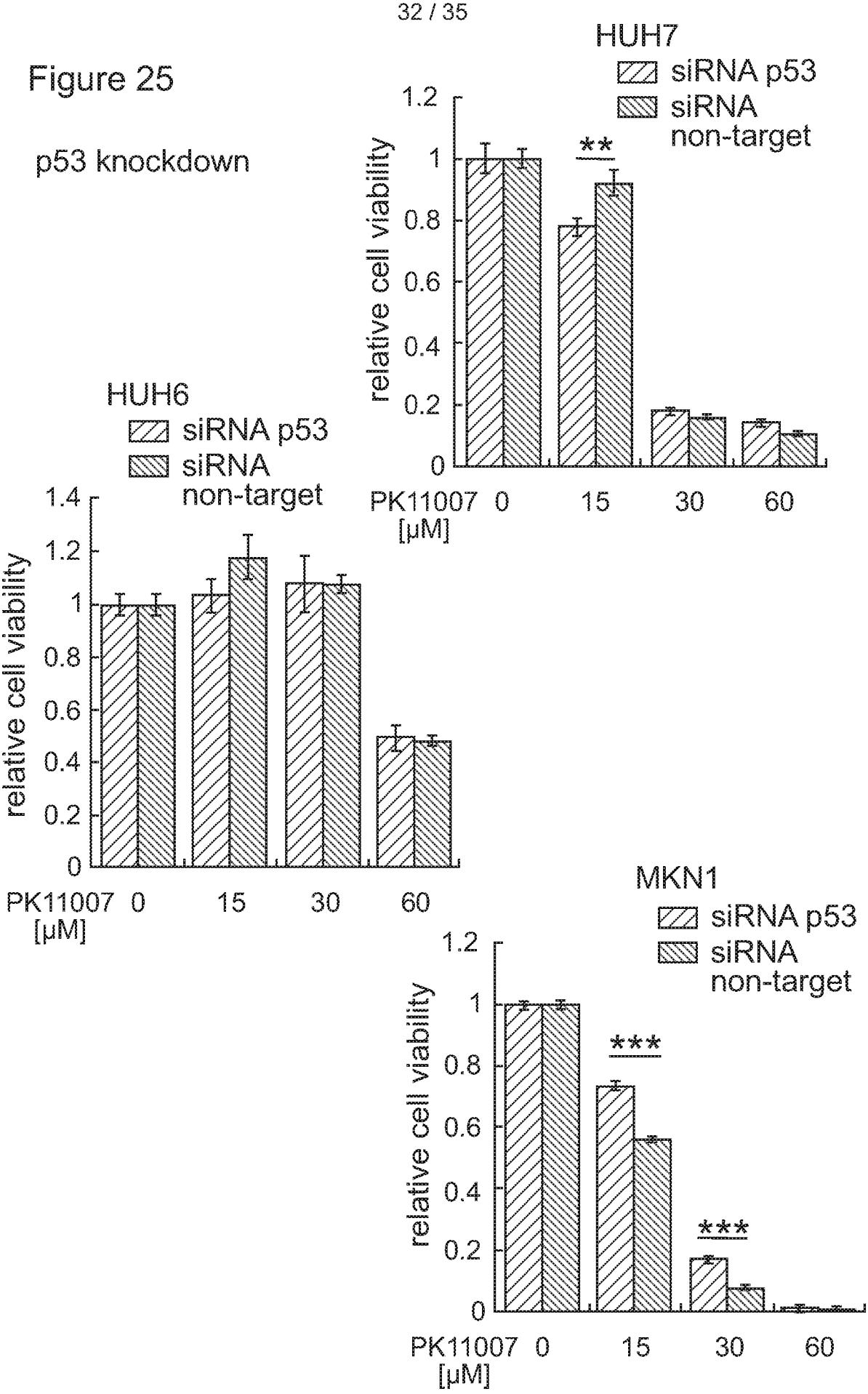


Figure 26

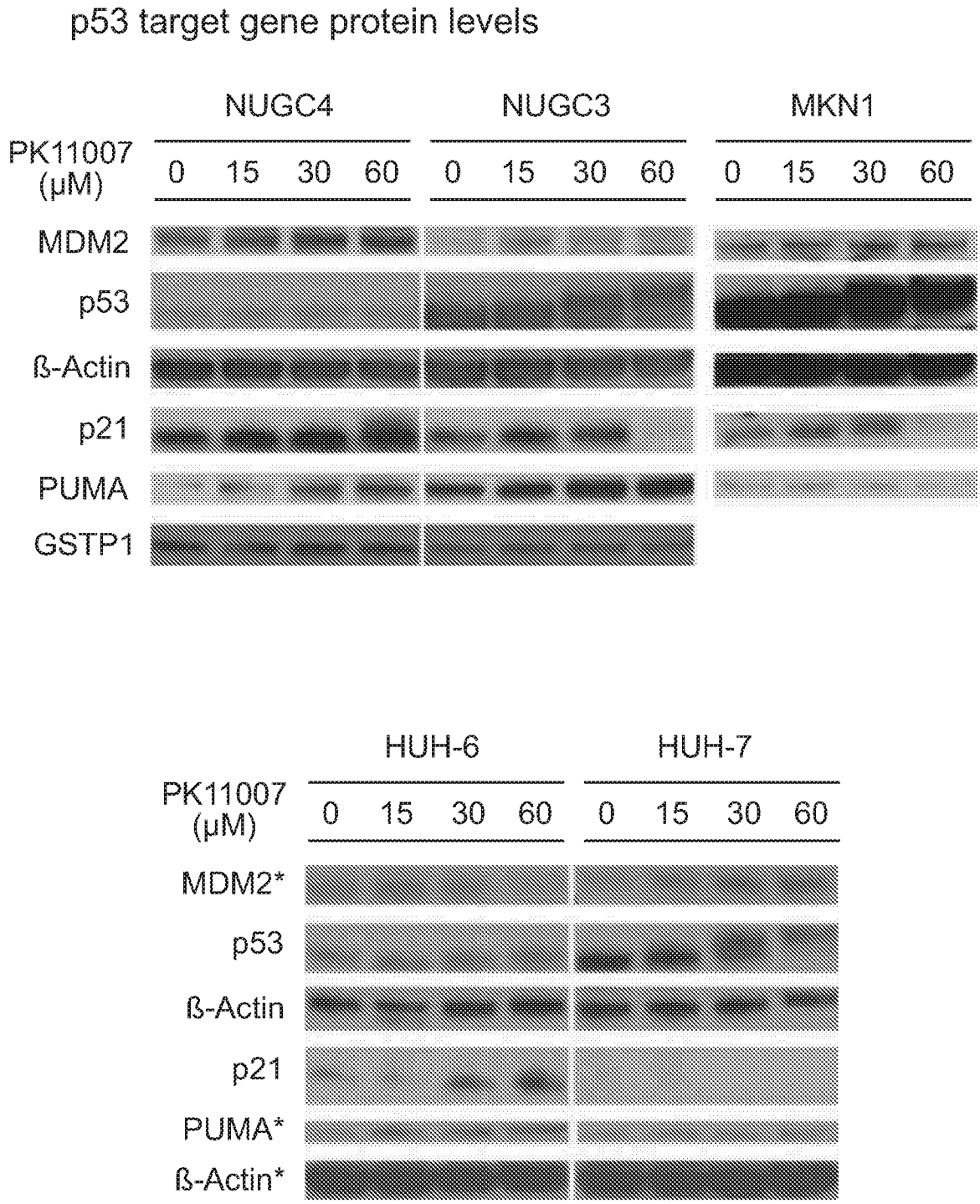

p53 target gene protein levels
NUGC4
NUGC3
MKN1
PK11007 (μM)
0 15 30 60 0 15 30 60 0 15 30 60
MDM2
p53
ß-Actin
p21
PUMA
GSTP1
HUH-6
HUH-7
PK11007 (μM)
0 15 30 60 0 15 30 60
MDM2*
p53
ß-Actin
p21
PUMA*
ß-Actin*

Figure 27

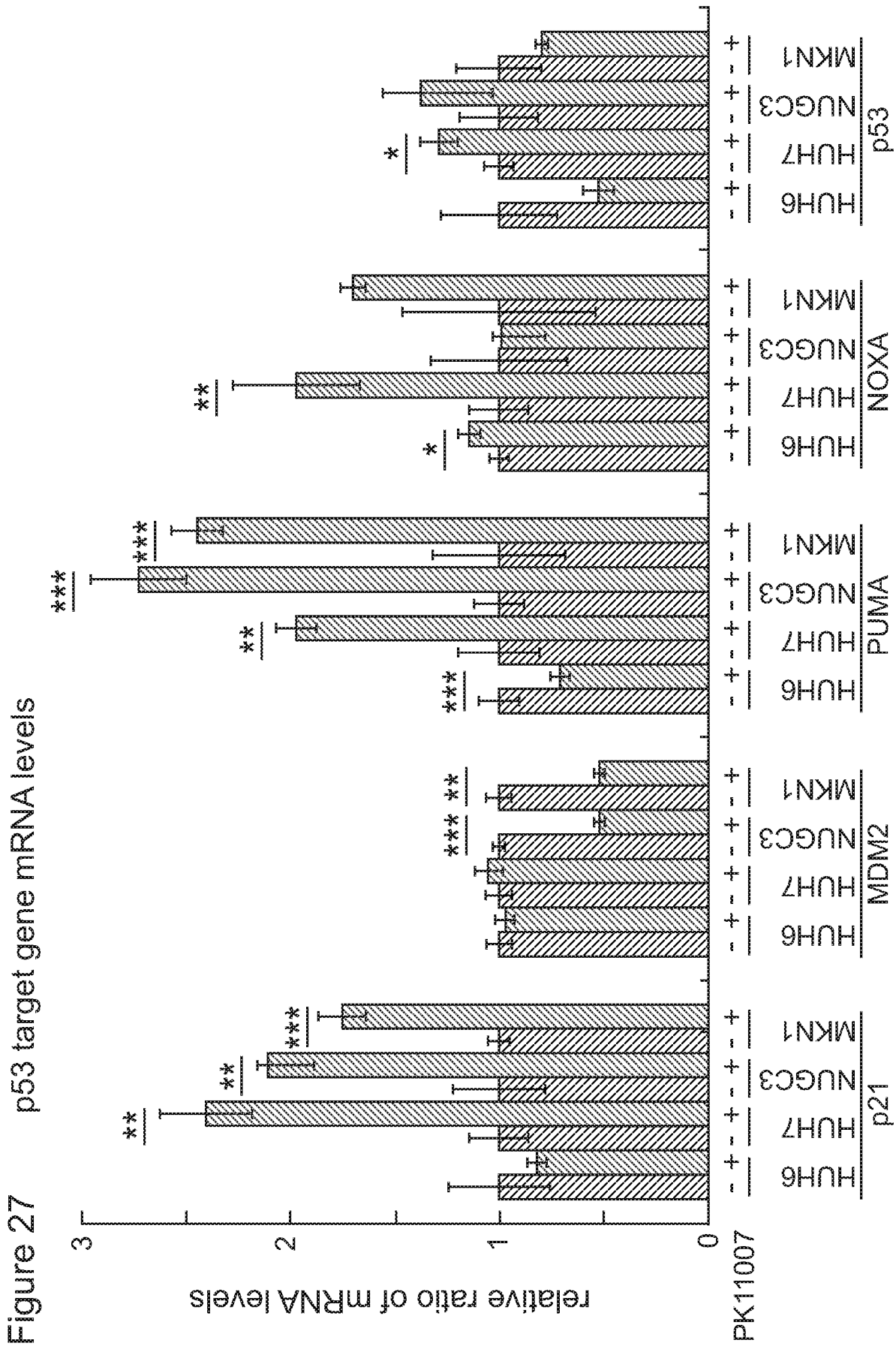
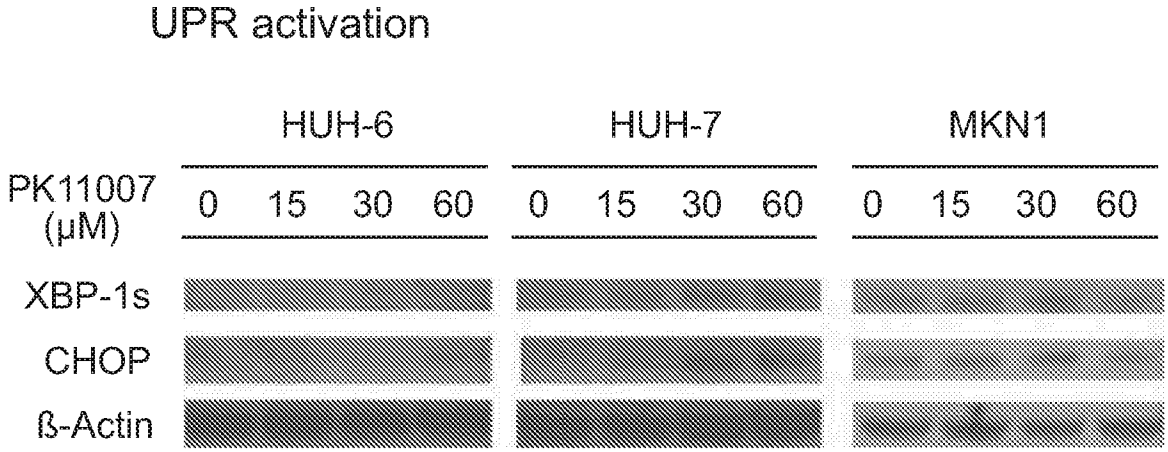

p53 target gene mRNA levels
relative ratio of mRNA levels
0
1
2
3
PK11007
p21
MDM2
PUMA
NOXA
p53
HUH6
HUH7
NUGC3
MKN1
**
***
*

Figure 28


UPR activation
HUH-6
HUH-7
MKN1
PK11007 (μM)
0 15 30 60
0 15 30 60
0 15 30 60
XBP-1s
CHOP
ß-Actin

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/052544

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D239/38 C07D417/12 A61K31/506 A61P35/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAREN G. ANTHONY ET AL: "New Classes of Alanine Racemase Inhibitors Identified by High-Throughput Screening Show Antimicrobial Activity against Mycobacterium tuberculosis", PLOS ONE, vol. 6, no. 5, 26 May 2011 (2011-05-26), page e20374, XP055305709, DOI: 10.1371/journal.pone.0020374 table 1; compound L206 -----	1-7
X	WO 2008/118626 A2 (BURNHAM INST MEDICAL RESEARCH [US]; PELLECCHIA MAURIZIO [US]) 2 October 2008 (2008-10-02) together with claim 54; page 26; compound III page 37; compounds C071-0352 page 19, line 35 ----- -/--	1-7

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

[X] See patent family annex.

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2016	07/10/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gutke, Hans-Jürgen

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/052544

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 033 195 A1 (NYEGAARD & CO AS [NO]) 5 August 1981 (1981-08-05) claims 1, 11 -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2016/052544

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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