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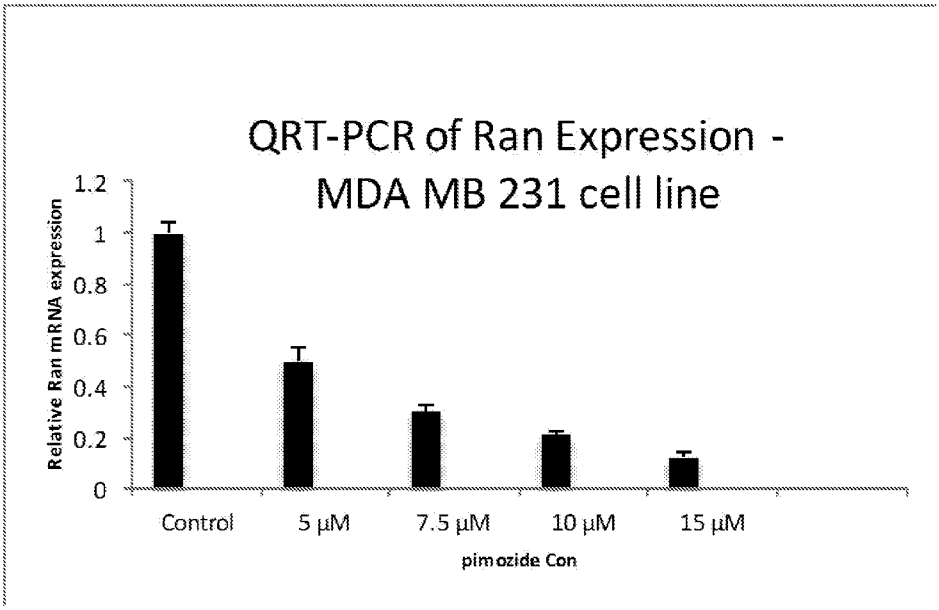
Figure 1A




(57) Abstract: The present invention relates to small molecule inhibitors of *RAN* at a transcriptional level and their use in the treatment of cancer, in particular cancers, such as triple negative breast cancer, that have been identified as overexpressing *RAN* or its protein product Ran. The inhibitors of the invention are particularly useful for the treatment of patients identified as having a tumour that overexpresses *RAN* or its protein product Ran. The invention also provides nanoparticle formulations of the small molecule inhibitors of the invention that avoid off target effects at the dopamine receptor and are thus suitable for selective cancer therapy.

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# INHIBITOR OF RAN AT TRANSCRIPTIONAL LEVEL FOR USE IN THE TREATMENT OF CANCER

## Field of Invention

The present invention relates to pimozide, or a pharmaceutically acceptable form thereof, for use in the treatment of cancers that overexpress *RAN* or its protein product Ran, in particular for the treatment of patients with tumours that have been identified as overexpressing *RAN* or its protein product Ran. The invention also provides novel nanoparticle formulations of pimozide, or a pharmaceutically acceptable form thereof, that are substantially free of dopaminergic effect when administered to a patient.

## Background of the Invention

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

The term cancer relates to a family of diseases characterised by abnormal cell growth. The abnormal new growth of cells is referred to as a neoplasm. The cells in a neoplasm usually grow more rapidly than normal cells and will continue to grow if left untreated. As they grow, neoplasms can impinge upon and damage adjacent structures, and can eventually compromise the survival of the host organism. Neoplasms can be benign or malignant (cancerous) and are often referred to as tumours. As used herein the term tumour is used to refer to malignant (cancerous) neoplasms. As a result, reference to the treatment of tumours and the treatment of cancer have the same meaning herein, while reference to breast cancer or breast tumour, and treatment thereof, refers to a cancer or tumour that originates in the breast, or treatment thereof, rather than a tumour that originates in another organ and that has spread to the breast through the process of metastasis. Tumours or cancers that originate in other organs or sites are referred to in a similar manner. Tumours that are derived from the process of metastasis are referred to as metastases or secondary tumours.

Hanahan and Weinburg ("The Hallmarks of Cancer". Cell 100 (1): 57–70) postulated that there are a number of traits or hallmarks that are common to the diseases known as cancer, namely (i) cancer cells stimulate their own growth; (ii) cancer cells resist inhibitory signals that might otherwise stop their growth; (iii) cancer cells resist their programmed cell death (they evade apoptosis); (iv) cancer cells can multiply indefinitely; (v) cancer cells stimulate the growth of blood vessels to supply nutrients to tumours (angiogenesis); and (vi) cancer cells invade local tissue and spread to distant sites (metastasis). Whether or not the full set of hallmarks, that have subsequently been supplemented ("Hallmarks of Cancer: The Next Generation" Cell 144

(5): 646–674), are exhibited by all cancers, it is apparent that targeting one or more of these hallmarks affords an opportunity to exert a selective killing effect on cancer cells.

Cancer cells are addicted to oncogenes or oncogenic pathways (Weinstein, Science 2002;297:63–4), which are usually uniquely present or hyperactivated, and are essential for tumour cell growth and survival. The two most frequently dysregulated signaling pathways in cancers are the phosphoinositide 3-kinase (PI3K)/Akt/mTORC1 and Ras/MEK/ERK [mitogen-activated protein/extracellular signal-regulated kinase (ERK; MEK)] pathways (Grant, J Clin Invest 2008;118:3003–6). These growth signaling pathways usually exert their ultimate effect by regulating the translocation of transcription factors into, or out of, the nucleus, thereby altering the transcriptome and consequently, the expressome (Kau & Silver, Drug Discov Today 2003;8:78–85). The activity of several oncogenic or tumour suppressive transcription factors, such as NF-κB, FOXO1, p53, and beta-catenin, and non-transcription factors, such as survivin, are regulated by their subcellular localization. Therefore, the uncoupling of nucleocytoplasmic transport from growth and survival signaling pathways has been suggested to be a potential target for cancer therapy.

Tumours or cancers are commonly referred to by the primary site of their occurrence, i.e. where the tumour first develops, and often also by reference to further characteristics, for example whether their growth is stimulated by hormones or whether they express certain receptors. Thus, primary tumours located in the breast are referred to breast cancers while tumours whose growth is stimulated by hormones such as estrogen, progesterone and testosterone are referred to as hormone dependent cancers. Breast cancers whose growth is stimulated by the presence of estrogen are known as estrogen receptor positive (ER+ve) breast cancers. Knowledge of tumour type can be exploited to select the best mode of therapy. For example, treatment of ER+ve tumours can involve estrogen deprivation, e.g. through blockage of estrogen biosynthesis with an aromatase inhibitor, or blocking of the signal derived from the interaction of an estrogen ligand with its receptor with a selective estrogen receptor modulator such as tamoxifen. These therapeutic interventions for the treatment of ER+ve tumours arrest the normal transcriptional activation of the estrogen receptor thereby blocking the estrogen stimulated growth of the tumour. Often, after presenting in an initial ER+ve state, breast cancer tumours progress to a hormone refactory state where their growth is independent of estrogen as, for example, through mutation, the estrogen receptor adopts a form in which it is transcriptionally activated even in the absence of estrogen, such tumours are also referred to as estrogen receptor negative (ER-ve) tumours. Similarly, breast cancers that express progesterone receptors and whose growth is sensitive to the presence of progesterone are referred to as progesterone positive (PR+ve) breast

cancers whilst those whose growth is independent of the expression of progesterone receptor are progesterone receptor negative (PR-ve) breast cancers.

Another common sub-type of breast cancer is HER2-positive breast cancer, a subset of breast cancers that over express human epidermal growth factor 2. A number of HER2 targeted therapies have been developed, examples include the anti-HER2 antibodies trastuzumab and pertuzumab and the antibody-drug-conjugate trastuzumab emtansine.

Breast cancers that are negative for ER, PR and HER2, i.e. those tumour types whose growth is independent of ER, PR or HER2 status, are commonly referred to as triple negative breast cancers. Patients suffering from triple negative breast cancers presently have the worst overall and disease free survival rates. Treatment options for patients with triple negative breast tumours are relatively limited (Ontilo et al, Clin Med Res. 2009 Jun; 7(1-2): 4–13) with the present treatment paradigms usually involving a combination of surgery and radiation as appropriate with cytotoxic chemotherapy with agents such as anthracyclines, taxanes or platinum chemotherapeutic agents, such as carboplatin, unless the patient has a tumour with a BRCA1 mutation. There is a clear clinical need to deliver new therapies for use in treating of such conditions to improve patient prognosis (Andre & Zielinski, Ann Oncol (2012) 23 (suppl 6): vi46-vi51). It is an object of the present invention to provide new therapies that are effective and are well tolerated for cancers such as triple negative breast cancer.

*RAN*, a member of the RAS Oncogene family, is a gene that encodes the GTP-binding nuclear protein Ran. Overexpression of *RAN* gene is observed in a number of cancers and this overexpression has been linked to poor patient prognosis. For example, *RAN* overexpression has been shown to correlate with increased aggressiveness of cancer cells *in vitro* and *in vivo* (Kurisetty et al, Oncogene 2008, 27, 7139-49), i.e. *RAN* overexpressing cancer cells are seen to grow rapidly and exhibit high metastatic potential. In contrast, silencing *RAN* by siRNA or shRNA reduced cell adhesion, migration and invasion *in vitro* and metastasis *in vivo*. Furthermore, *in vitro* studies have demonstrated that silencing the *RAN* gene with SiRNA or shRNA induces a greater degree of apoptosis in cancer cells relative to that induced in normal cells and in activated KRas–mutant cells relative to their isogenic K-Ras wild-type counterparts. Cancer cells are observed to be more sensitive to changes in *RAN* status than their normal counterparts. *RAN* silencing has also been observed to promote apoptosis in cancer cells with mutations that correlate with activation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK signalling pathways (Yuen et al, Clin Cancer Res 2011; 18(2); 1-12). As a result, *RAN* has been suggested as a potential therapeutic target for cancer phenotypes in which the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways are activated.

Ran (Ras-related nuclear) protein, a 25-kDa protein product encoded by RAN gene, is a G-protein GTPase that cycles between a GDP-bound (RanGDP) and a GTP-bound (RanGTP) state that regulates nucleocytoplasmic transport, mitotic spindle fibre assembly and post-mitotic nuclear envelope dynamics.

Ran exists in a different conformation depending on whether it is bound to GTP or GDP. In its GTP bound state, Ran is capable of binding karyopherins (importins and exportins) a set of proteins that are involved in importing and exporting molecules between the nucleus and the cytoplasm of a eukaryotic cell. Importins release a molecular cargo upon binding to RanGTP in the nucleus, while exportins must bind RanGTP in the nucleus to form a ternary complex with their export cargo in order to transport the cargo to the cytoplasm.

The dominant nucleotide binding state of Ran depends on whether it is located in the nucleus (RanGTP) or the cytoplasm (RanGDP), with RanGTP being formed inside the nucleus through interaction of Ran with its specific guanine nucleotide exchange factor (GEF), regulator of chromosome condensation 1 referred to herein as RCC1, which catalyses the exchange of GDP for GTP on the nucleotide binding pocket of Ran. Hydrolysis of RanGTP to RanGDP in the cytoplasm by RanGAP and RanBP1 releases energy and causes the ternary complex of Ran, exportin and cargo to dissociate thus releasing the cargo exported from the nucleus. Cytoplasmic RanGDP is in turn imported into the nucleus by the small protein NTF2 (Nuclear Transport Factor 2), where RCC1 can then catalyse exchange of GDP for GTP on Ran and complete the Ran cycle.

During mitosis, the Ran cycle is involved in mitotic spindle assembly and nuclear envelope reassembly after the chromosomes have been separated. During prophase, the steep gradient in RanGTP-RanGDP ratio at the nuclear pores breaks down as the nuclear envelope becomes leaky and disassembles. RanGTP concentration stays high around the chromosomes as RCC1 stays attached to chromatin as the nucleoporin RanBP2 (Nup358) and Ran GTPase activating protein (RanGAP) move to the kinetochores where they facilitate the attachment of spindle fibres to chromosomes. Moreover, RanGTP promotes spindle assembly by mechanisms similar to mechanisms of nuclear transport: the activity of spindle assembly factors such as NuMA and TPX2 is inhibited by the binding to importins. By releasing importins, RanGTP activates these factors and therefore promotes the assembly of the mitotic spindle. In telophase, RanGTP hydrolysis and nucleotide exchange are required for vesicle fusion at the reforming nuclear envelopes of the daughter nuclei.

There is a growing awareness that *RAN* is a potential target for cancer therapy, in particular breast cancer therapy. Studies have shown that breast cancer patients whose primary tumours have a higher percentage of malignant cell nuclei that stain for Ran have a shorter median survival time than those with less than 1% of cell nuclei that stain for Ran (P <0.001). When analyzed alongside tumour size, grade, and lymph node involvement using Cox-regression analysis, Ran nuclear staining was independently associated with patient survival time [relative risk (RR) 12.53, 95% CI: 3.95–39.758, P < 0.001] (Yuen HF, El-Tanani M, *et al. J Natl Cancer Inst*. 2013;105(7):475-88 & Yuen HF, El-Tanani M, *et al. Clin Cancer Res*. 2012;18(2):380-91).

Analysis of the GEO breast cancer data set f (200 patients (GSE2034)) indicated that a high level of Ran significantly correlates to shorter survival time in patients with PIK3CA mutation gene signature (P = 0.018), but not in those with PIK3CA wild-type gene signature (P = 0.186) (Yuen HF, El-Tanani M, *et al. J Natl Cancer Inst*. 2013;105(7):475-88 & Yuen HF, El-Tanani M, *et al. Clin Cancer Res*. 2012;18(2):380-91).

Stable transfection of non-invasive mammary cells (R37 and MCF-10A) with an expression vector for Ran (R37-Ran and MCF-10A-Ran) was found to induce an invasive/metastatic phenotype *in vitro* and the development of metastases *in vivo (Kurisetty VV, El-Tanani MK et al. Oncogene. 2008;27(57):7139-49*). Stable transfection of invasive cells (MDA-MB-231, R37-OPN and R37-RAN) with siRNA directed at Ran specifically inhibits the invasive/metastatic phenotype *in vitro* and *in vivo* (Kurisetty VV, El-Tanani MK et al. Oncogene. 2008;27(57):7139-49, Yuen HF, El-Tanani M, et al. *J Natl Cancer Inst*. 2013;105(7):475-88 & Yuen HF, El-Tanani M, et al. *Clin Cancer Res*. 2012;18(2):380-91. In addition, inhibition of Ran expression in tumour cell lines was found to cause abnormal mitotic spindle formation, mitochondrial dysfunction, and apoptosis. Use of siRNA/shRNAi or expression vector for Ran dominant negative (G19V/Q69L) mutant was found to lead to an anti-proliferative and/or anti-invasive phenotype in multiple models including breast and lung (Kurisetty W, El-Tanani MK *et al. Oncogene*. 2008;27(57):7139-49).

Ran silencing was found to induce a significant apoptotic response in GR5 c-Met amplified cells in the presence of gefitinib, but not in HCC827 parental cells ($p$ <0.01), suggesting that Ran silencing may be used as a modulator of chemosensitivity (Yuen HF, El-Tanani M, *et al. J Natl Cancer Inst.* 2013;105(7):475-88).

Silencing Ran expression induced higher apoptosis in breast cancer MDA-MB-231 than immortalized MCF10a cells, and c-Met amplified cells compared to their isogenic *c-Met* wild-

type counterparts, respectively (Yuen HF, El-Tanani M, *et al. J Natl Cancer Inst.* 2013;105(7):475-88).

It is an object of the present invention to provide small molecules that inhibit *RAN* at the transcriptional level and their use in the treatment of cancers that overexpress *RAN* or its protein product Ran, for instance in patients who have been identified as having a cancer that overexpresses *RAN* or its protein product Ran. Diseases overexpressing *RAN* include but are not limited to certain breast, lung, prostate, ovarian, blood, brain and renal cancers and include those cancers currently associated with poor patient prognosis such as triple negative breast cancer.

## Summary of the Invention

In a first aspect of the invention there is provided Pimozide, or a pharmaceutically acceptable form thereof, for use as an inhibitor of *RAN* at a transcriptional level. In preferred embodiments, the Pimozide for use as an inhibitor of *RAN* at a transcriptional level, is for use in the treatment of cancer, for example a cancer that overexpresses *RAN* or its protein product Ran. In most preferred cases the Pimozide for use as an inhibitor of *RAN* at a transcriptional level in the treatment of cancer, is for use in patients identified as having a cancer that overexpresses *RAN* or its protein product Ran. As used herein and above, a cancer that overexpresses *RAN*, may overexpress Ran mRNA and/or Ran protein and may be identified, for example, by establishing that elevated levels of Ran mRNA and/or Ran protein are present in the cancer cells.

In embodiments, pimozide is used as an inhibitor of *RAN* in combination with an inhibitor of an epidermal growth factor receptor (EGFR) tyrosine kinase such as the small molecules gefitinib, erlotinib, afatinib, brigatinib, icotinib, or osimertinib or the antibodies cetuximab, panitumumab, zalutumumab, nimotuzumab, or matuzumab. Molecules and antibodies that inhibit epidermal growth factor receptor (EGFR) tyrosine kinase are generally referred to herein as EGFR inhibitors. In such cases the co-administration of pimozide and an EGFR inhibitor may be simultaneous, sequential or separate. It has been demonstrated that the combination of pimozide and an EGFR inhibitor has a synergistic effect against cancer cell proliferation. It has also been shown that pimozide can restore sensitivity of gefitinib resistant cancer cells to gefitinib treatment therefore providing a new therapeutic approach for the treatment of patients who have progressed on EGFR inhibitor therapy and, potentially, a new mechanism to prevent development of EGFR inhibitor resistance.

Identification of the patients indicated for pimozide treatment, i.e. those having a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA, may be achieved by obtaining a sample from the candidate patient, for example a tumour biopsy or a blood sample, and analysing the sample for the presence of *RAN* or a biomarker for *RAN* expression. Alternatively, or additionally, the patient identification may involve testing for mutation status of a tyrosine kinase such as EGFR.

Analysis of a tumour biopsy for identifying a patient having a *RAN* overexpressing tumour can, for example, be carried out by immunohistochemical means. Thus, a tumour biopsy sample can be stained with a stain comprising a *RAN* selective antibody linked to a visualising means such as an enzyme or fluorophore. The stained tumour biopsy can then be categorised as a *RAN* overexpressing tumour or a non-*RAN* overexpressing tumour. This categorisation can be done by a visual or automated scoring. Testing for tyrosine kinase mutation status can be by the methods available in the art, for example the commercially available testing kits produced by Genzyme, QIAGEN and Argenomics S.A. for predicting response to the various tyrosine kinase inhibitors.

Analysis of a patient blood sample to determine whether a patient has a *RAN* overexpressing tumour may be performed by isolating the serum exosome and then carrying out an ELISA or quantitative real time PCR (q RT-PCR) analysis to provide the necessary tumour/patient classification.

In preferred embodiments, the patient is a human patient. In embodiments the patient is a non-human mammal and may be selected from the members of the the Canidae, Felinae, Bovidae, Equidae, Suidae, Camelini and Cervidae families.

In some embodiments, the pimozide for use is administered in a dosage of from 1 to 5000 mg/day.

In some embodiments, the pimozide for use is in the form of a nanoparticle formulation, optionally the nanoparticle formulation comprises a poly(lactic-co-glycolic acid) copolymer, for example a poly(lactic-co-glycolic acid) / polyethylene glycol block copolymer.

In a second aspect the invention relates to a nanoparticle formulation of Pimozide or a pharmaceutically acceptable form thereof. In a preferred embodiment the nanoparticle formulation comprises a poly(lactic-co-glycolic acid) / polyethylene glycol block copolymer.

The invention also relates to a method of treatment of a cancer that overexpresses *RAN*, or its protein product Ran, comprising a step of administering to a patient in need thereof an

effective amount of pimozide or a pharmaceutically acceptable form thereof. This method of treatment may further comprise a step of testing a sample obtained from said patient to determine whether said patient has a *RAN* overexpressing cancer, e.g. a cancer that overexpresses Ran or Ran mRNA, prior to said administration step.

The invention also provides a method of treatment of a cancer that overexpresses *RAN*, or its protein product Ran, comprising the step of administering to a patient who has been identified as having a cancer that overexpresses *RAN*, or its protein product Ran, an effective amount of pimozide or a pharmaceutically acceptable form thereof. This method of treatment may further comprise a step of testing a sample obtained from said patient to determine whether said patient has a *RAN* overexpressing cancer prior to said administration step.

The invention also provides a method of treatment of a cancer that overexpresses *RAN*, or its protein product Ran, comprising the steps of selecting a patient who has a cancer that overexpresses *RAN* or its protein product Ran, and administering an effective amount of pimozide or a pharmaceutically acceptable form thereof to that patient. This method of treatment may further comprise a step of testing a sample obtained from said patient to determine whether said patient has a *RAN* overexpressing cancer prior to said administration step.

The invention also provides a method of treatment for cancer in a patient diagnosed as having a cancer characterised by an overexpression of *RAN* or its protein product Ran involving administration to a patient in need thereof an effective amount of pimozide. The cancer characterised by an overexpression of *RAN* may be triple negative breast cancer. The cancer characterised by an overexpression of *RAN* may be lung cancer, and in particular lung cancers that have previously been treated with a tyrosine kinase inhibitor, for example wherein the previous treatment involved administration of an tyrosine kinase inhibitor such as an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor such as gefitinib, erlotinib, afatinib, brigatinib, icotinib or osimertinib to a lung cancer patient and wherein the disease no longer responds to the treatment with the EGFR inhibitor. Such methods may involve co-administration of the previously used tyrosine kinase inhibitor with pimozide.

The invention also provides a method of treatment for cancer in a patient diagnosed as having a cancer characterised by an overexpression of a tyrosine kinase, such as a mutated tyrosine kinase, such as epidermal growth factor receptor (EGFR) tyrosine kinase, involving administering pimozide in combination with an inhibitor of the tyrosine kinase that is overexpressed, for example in a cancer with a mutation in the EGFR tyrosine kinase domain, an EGFR inhibitor may be used. In such methods, the administration of pimozide and an

EGFR inhibitor may be simultaneous, sequential or separate. Advantageously the combination of pimozide and an EGFR inhibitor may prevent or substantially delay the emergence of resistance to the EGFR inhibitor associated with EGFR inhibitor monotherapy. Advantageously, the combination of pimozide and an EGFR inhibitor may resensitize a tumour to treatment with an EGFR inhibitor to which the patient no longer responds as a monotherapy.

In a further aspect the invention relates to the use of pimozide, or a pharmaceutically acceptable form thereof, for the manufacture of a medicament for the treatment of cancer, optionally wherein the cancer overexpresses *RAN* or its protein product Ran or Ran mRNA or wherein the cancer is indicated for tyrosine kinase inhibitor therapy, for example wherein the cancer has a mutated and/or overactive epidermal growth factor receptor. In embodiments the resultant medicament is provided in a package with instruction for use in the treatment of a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA.

In a further aspect the invention provides a package comprising pimozide or a pharmaceutically acceptable form thereof and instructions for its use in the treatment of a cancer that overexpresses *RAN* or its protein product Ran.

**Description of Figures**

In order that the invention can be fully understood, the invention and embodiments thereof are described with reference to the following Figures, which are not intended to limit the scope of the invention.

Figure 1 illustrates the effects of pimozide treatment on the relative Ran mRNA expression in (A) MDA MB-231 human breast cancer cells and (B) A549 lung cancer cells.

Figure 2A illustrates the effect of pimozide treatment on *RAN* transcription in a dual luciferase reporter assay in MDA MB-231 cells. Figure 2B shows the effect of Pimozide treatment on Myc expression in MDA MB-231 cells.

Figure 3 shows the effect of pimozide treatment on Ran GTPase activity in MDA MB-231 cells treated with 10 μM and 15 μM of pimozide vs control

Figure 4 shows A) the effect on MDA-MB 231 human breast cancer cells, A549 human lung cancer cells and MCF10A mammary epithelial cells of incubation with pimozide for 48 hours and B) the effect of pimozide in provoking DNA damage response (i) control, ii) pimozide treatment and in promoting DNA double strand breaks (iii) control, iv) pimozide treatment).

Figure 5 shows A) bioluminescent imaging of tumours and metastases in a murine model in control (Group 2, G2-NT), early treatment (Group 3, G3-ET) and late treatment (Group-4, G4-TL) cohort; B) quantification of tumour volumes at the end of the study in each cohort; & C) number of metastases in each cohort.

Figure 6 shows results from histological staining of tumours obtained from the study of figure 5 for haematoxylin & eosin (H & E, row 1), Ki67(row 2); cleaved caspase-3(row 3) and Ran (row 4)) –in untreated animals (UT, first column), early treatment (ET, second column) and late treatment (LT, third column) cohorts respectively.

Figure 7 shows histological staining of cells treated 7.5 μM pimozide or control for A) paxillin, actin and merged image in MDA MB-231; B) myosin IIA, actin and merged image in MDA MB-231 and C) Arp3, actin and merged image in A549 cells.

Figure 8 A) RT-PCR of EMT markers Vimentin and Zo-1 24 hours after treatment with pimozide at 7.5 μM and 10 μM, showing a reduction of expression in agarose gel of both vimentin and Zo-1. β-actin was used as control of expression. B) Western blot analysis of protein expression of Snail, Vimentin and N-Cadherin after 24 hours of pimozide (RI) treatments. C) Immunohistochemistry of vimentin expression in untreated mice (PBS) and treated tumor xenograft sections with pimozide (a representative of 10 sections per group). D) Relative gene expression of EMT markers in MDA-MB-231 cell lines treated with pimozide (RI) at 7.5 μM after 24 hours, showing a decrease of mRNA gene expression in Ncadherin (Ncad), Vimentin (Vim), Snail, slug and twist compared to untreated control (DMSO).

Figure 9 shows cell viability (MDA MB-231 cells) at 24h, 48h, 72h and 96h following treatment with control, empty nanoparticles, free pimozide and pimozide formulated as 5%-PEG-PLGA nanoparticles (NPs).

Figure 10 shows flow cytometry data obtained from cells after treatment with A) control, B) FITC dye containing nanoparticles C) FITC dye/pimozide PCL nanoparticles and D) FITC dye/pimozide 5%-PEG-PLGA nanoparticles.

Figure 11 shows cellular uptake of pimozide labelled nanoparticles by MDA MB-231 cells.

Figure 12 shows the effect on HCC827 human cancer cell survival following treatment with A) gefitinib, B) pimozide and C) various concentrations of pimozide in combination with 300 nM gefitinib.

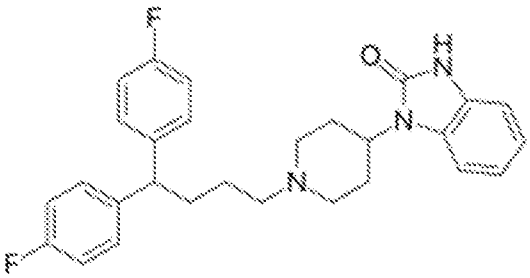
Figure 13 shows the effect on HCC827 GR5 (gefitinib resistant) human cancer cell survival following treatment with A) gefitinib B) pimozide and C) various concentrations of pimozide in combination with 300 nM gefitinib.

**Detailed description of the invention**

The present invention relates to Pimozide, or a pharmaceutically acceptable form thereof, for use in the treatment of a cancer that overexpresses RAN or its protein product Ran, for example in patients identified as having a cancer that overexpresses RAN or Ran.

Ran, a protein product of the gene RAN, is a member of the Ras superfamily that regulates nucleocytoplasmic transport, mitotic spindle fibre assembly and post-mitotic nuclear envelope dynamics. Ran acts as a molecular switch through a GTP-GDP cycle in which the conversion between GTP-bound and GDP-bound conformations controls its interaction with different effectors. RanGTP is formed inside the nucleus by interaction of Ran with its specific guanine nucleotide exchange factor (GEF), referred to herein as RCC1, which catalyses the exchange of GDP for GTP (and vice versa) on the nucleotide binding pocket of Ran. RAN, and its protein product Ran are known to be overexpressed in a number of cancers including those in which the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways are activated. Diseases overexpressing Ran include but are not limited to certain breast, lung, prostate, ovarian, blood, brain and renal cancers and include those cancers currently associated with poor patient prognosis such as triple negative breast cancer.

Pimozide, 1-[1-[4,4-bis(4-fluorophenyl)butyl]-4-piperidinyl]-1,3-dihydro-2H-benzimidazole-2-one, is an antipsychotic drug of the diphenylbutylpiperidine class that acts as an antagonist of dopamine D2 receptors at nanomolar concentrations. Pimozide has also been identified as an antagonist of the σ opioid receptor. There is evidence that the dopamine D2 receptor and σ opioid interactions of pimozide are important factors in delivering antipsychotic activity. In addition, experimental data suggests that pimozide is a weak protein kinase C inhibitor. At present, pimozide is clinically used for the treatment of schizophrenia and chronic psychosis, Tourette's syndrome and resistant tics and is marketed under various trade names such Orap®.



Pimozide, Formula (I)

A number of reports have indicated that pimozide may exert an anti-cancer effect *in vitro* and *in vivo* models of cancer (see e.g. Strobl JS *et al*, *Breast Cancer Res Treat* **51**:83–95). Prior to the present invention however, the effects of pimozide on cancers that overexpress *RAN* and its protein product Ran were not known. Furthermore, until the present invention it was not known that pimozide could inhibit *RAN* at a transcriptional level. As described herein, the identification of pimozide as an inhibitor of *RAN* at a transcriptional level, thereby suppressing the downstream effects of the *RAN* gene, provides a new and advantageous option for therapy of patients with cancers that overexpress *RAN* or its protein product Ran. The present invention advantageously allows identification of groups of cancer patients that are indicated for pimozide treatment and advantageously allows a therapeutic intervention for such patients that is targeted to the genetic profile of their cancer. The present invention thus provides for improved therapeutic outcomes for cancer patients with cancers that overexpress *RAN* and its protein product Ran.

Pharmaceutically acceptable forms of pimozide include prodrugs of pimozide, that is to say compounds which break down and/or are metabolised *in vivo* to provide an active compound of formula (**I**). General examples of prodrugs include simple esters, and other esters such as mixed carbonate esters, carbamates, glycosides, ethers, acetals and ketals. Prodrug motifs may, for example, be directly introduced at the benzimidazole NH position of pimozide.

Pharmaceutically acceptable forms of pimozide include metabolites of pimozide, in particular metabolites that retain an inhibitory effect on *RAN* transcription. A metabolite, as employed herein, is a compound that is produced *in vivo* from the metabolism of pimozide, such as, without limitation, oxidative metabolites.

Pharmaceutically acceptable forms of pimozide also include deuterated forms of pimozide. Deuterated forms of pimozide are derivatives of pimozide in which one or more aliphatic or aromatic hydrogen atom is substituted for a deuterium atom. For example, a hydrogen atom

at a site susceptible to metabolism may be replaced by a deuterium atom to reduce metabolism at that site.

Pharmaceutically acceptable forms of pimozide include formulations of pimozide, for example formulations that exhibit a pharmacological profile that is different to the clinical formulations presently in use for treatment of neurological conditions.

The present invention also relates to a pimozide, for use in combination with a tyrosine kinase inhibitor, for example a receptor tyrosine kinase inhibitor such as an inhibitor of EGFR tyrosine kinase or a non-receptor tyrosine kinase inhibitor such as a Bcr-Abl kinase inhibitor, for the treatment of cancer. Other receptor tyrosine kinases inhibitors that may be used in combination with a pimozide include inhibitors of PDGFR and FGFR kinases. Other non-receptor tyrosine kinases inhibitors that may be used in combination with a pimozide include inhibitors of SRC, FAK and JAK. As can be seen from the data provided in Figures 12 and 13 co-administration of pimozide with the receptor tyrosine kinase inhibitor gefitinib or a non-receptor tyrosine kinase inhibitor dasatinib provides a synergistic, i.e. better than additive, effect against the survival of human lung cancer cells.

Examples of tyrosine kinase inhibitors that may be used in combination with a Ran inhibitor for the treatment of cancer include afatinib (Giotrif™), axitinib (Inlyta™), bosutinib (Bosulif™), crizotinib (Xalkori™), dasatinib (Sprycel™), erlotinib (Tarceva), gefitinib (Iressa™), osimertinib (Tagrisso™), imatinib (Glivec™), lapatinib (Tyverb™), nilotinib (Tasigna™), pazopanib (Votrient™), regorafenib (Stivarga™), sorafenib (Nexavar™), sunitinib (Sutent™) and ibrutinib (Imbruvica™). The co-administration of pimozide and a tyrosine kinase inhibitor may be simultaneous, sequential or separate.

In some preferred embodiments pimozide is used as an inhibitor of *RAN* in combination with an inhibitor of an epidermal growth factor receptor (EGFR) tyrosine kinase such as the small molecules gefitinib, erlotinib, afatinib, brigatinib, icotinib, or osimertinib or the antibodies cetuximab, panitumumab, zalutumumab, nimotuzumab, or matuzumab. Molecules and antibodies that inhibit epidermal growth factor receptor (EGFR) tyrosine kinase are generally referred to herein as EGFR inhibitors. In such cases the co-administration of pimozide and an EGFR inhibitor may be simultaneous, sequential or separate. It has been demonstrated that the combination of pimozide and an EGFR inhibitor, namely gefitinib, has a synergistic effect against cancer cell proliferation (see Figures 12 and 13). It has also been shown that pimozide can restore sensitivity of gefitinib resistant cancer cells to gefitinib treatment therefore providing a new therapeutic approach for the treatment of patients who have

progressed on EGFR inhibitor therapy and, potentially, a new mechanism to prevent development of EGFR inhibitor resistance (see Figure 13).

Identification of the patients indicated for combination therapy with pimozide and a tyrosine kinase inhibitor may involve testing for mutation status of a tyrosine kinase such as EGFR. Testing for tyrosine kinase expression/mutation status can be by the methods available in the art, for example the commercially available testing kits produced by Genzyme, QIAGEN and Argenomics S.A. for predicting response to the various tyrosine kinase inhibitors.

**Methods of treatment**

It is an aspect to provide pimozide, or a pharmaceutically acceptable form thereof, as an inhibitor of RAN transcription for use as a medicament.

In one aspect, the present invention provides methods for treatment or alleviation of a cancer over expressing RAN, or its protein product Ran, in the tissue of one or more organs as mentioned herein, comprising the step of administering pimozide or a pharmaceutically acceptable form thereof to a patient in need thereof. In embodiments the method treatment is for patients with a cancer that have been diagnosed as having a cancer that overexpresses RAN or Ran or Ran mRNA.

Such methods according to the present invention in one embodiment comprise one or more steps of administration or release of an effective amount of pimozide or a pharmaceutically acceptable form thereof, or a pharmaceutical composition comprising pimozide or a pharmaceutically acceptable form thereof, to an individual in need thereof. In one embodiment, such steps of administration or release according to the present invention is simultaneous, sequential or separate.

An individual in need as referred to herein, is in one embodiment an individual that benefits from the administration of a pimozide or a pharmaceutically acceptable form thereof, in particular an individual with a cancer that overexpresses *RAN* or its protein product Ran. Such an individual in one embodiment suffers from a malignant neoplasm (tumour) in the tissue of one or more organs. In preferred examples the tumour(s) overexpresses Ran. In embodiments the cancer is selected from cancers of the breast, lung, ovary or kidney. In embodiments the cancer is one in which the PI3K/Akt/mTORC1 and/or Ras/MEK/ERK pathways are activated. In embodiments the cancer is triple negative breast cancer.

The individual is in one embodiment any human being, male or female, infant, middle-aged or old.

Identification of the patient to be treated in the methods of the invention can be achieved in a number of ways. For example, the identification may involve analysis of a sample obtained from a patient, for example a blood sample or a tumour biopsy sample. Analysis of a patient blood sample may be performed by isolating the serum exosome and then carrying out an ELISA or quantitative real time PCR (q RT-PCR) analysis to provide the necessary tumour/patient classification.

Analysis of a tumour biopsy for identifying a patient having a *RAN* overexpressing tumour can, for example, be carried out by immunohistochemical means. Thus a tumour biopsy sample can be stained with a stain comprising a *RAN* selective antibody linked to a visualising means such as an enzyme or fluorophore. The stained tumour biopsy can then be categorised as a *RAN* overexpressing tumour or a non-*RAN* overexpressing tumour. This categorisation may be performed by a visual or automated scoring. Preliminary work has demonstrated that scoring of Ran immunohistochemically stained tumour biopsy sample slides can be performed under a light microscope and that an assessment of the % of tumour cells staining over the whole section provides a method to identify tumours that overexpress *RAN*.

The invention also provides a method of treatment for cancer in a patient diagnosed as having a cancer characterised by an overexpression of *RAN* or its protein product Ran involving administration to a patient in need thereof an effective amount of pimozide. The cancer characterised by an overexpression of *RAN* may be triple negative breast cancer. The cancer characterised by an overexpression of *RAN* may be lung cancer, and in particular lung cancers that have previously been treated with a tyrosine kinase inhibitor, for example wherein the previous treatment involved administration of an tyrosine kinase inhibitor such as an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor such as gefitinib, erlotinib, afatinib, brigatinib, icotinib or osimertinib to a lung cancer patient and wherein the disease no longer responds to the treatment with the EGFR inhibitor. Such methods may involve co-administration of the previously used tyrosine kinase inhibitor with pimozide.

The invention also provides a method of treatment for cancer in a patient diagnosed as having a cancer characterised indicated for tyrosine kinase therapy, by an overexpression of an tyrosine kinase, such as a mutated epidermal growth factor receptor (EGFR) tyrosine kinase, involving administering pimozide in combination with an inhibitor of the tyrosine kinase that is overexpressed, for example in a cancer with a mutation in the EGFR tyrosine kinase domain, an EGFR inhibitor may be used. In such methods the administration of pimozide and an EGFR inhibitor may be simultaneous, sequential or separate. Advantageously the combination of pimozide and an EGFR inhibitor may prevent or substantially delay the emergence of resistance to the EGFR inhibitor associated with EGFR inhibitor monotherapy.

Advantageously the combination of pimozide and an EGFR inhibitor may resensitize a tumour to treatment with an EGFR inhibitor to which the patient no longer responds as a monotherapy.

The terms "treatment" and "treating" as used herein refer to the management and care of a patient for the purpose of combating a condition, disease or disorder. The term is intended to include the full spectrum of treatments for a given condition from which the patient is suffering, such as administration of pimozide or a pharmaceutically acceptable form thereof for the purpose of: alleviating or relieving symptoms or complications; delaying the progression of the condition, partially arresting the clinical manifestations, disease or disorder; curing or eliminating the condition, disease or disorder; and/or preventing or reducing the risk of acquiring the condition, disease or disorder, wherein "preventing" or "prevention" is to be understood to refer to the management and care of a patient for the purpose of hindering the development of the condition, disease or disorder, and includes the administration of the active compounds to prevent or reduce the risk of the onset of symptoms or complications. The patient to be treated is preferably a mammal, in particular a human being. Treatment of animals, such as mice, rats, dogs, cats, cows, horses, sheep and pigs, is, however, also within the scope of the present invention. The patients to be treated according to the present invention can be of various ages, for example, adults, children, children under 16, children age 6-16, children age 2-16, children age 2 months to 6 years or children age 2 months to 5 years.

The invention is thus, in one embodiment, directed to pimozide or a pharmaceutically acceptable form thereof for use in the treatment of cancer in the tissue of one or more organs of a mammal. In one embodiment said treatment is ameliorative and/or curative. In one embodiment, said mammal is a human (homo sapiens).

When referring to the tissue of one or more organs, said organ is in one embodiment selected from the group consisting of breast, lung, ovarian, prostate, blood, brain and renal cancers

**Formulations**

Pimozide or a pharmaceutically acceptable form thereof for use in the treatment of cancer that overexpresses *RAN*, or its protein product Ran, may be provided as a pharmaceutical composition comprising pimozide or a pharmaceutically acceptable form thereof in combination with one or more pharmaceutically acceptable diluents or carriers. The formulation may be a formulation currently used in the clinic for pimozide or may be a new formulation specifically adapted to the purpose of treating *RAN* overexpressing cancers.

Diluents and carriers may include those suitable for parenteral, oral, topical, mucosal and rectal administration.

The present invention also provides a process for preparing such a pharmaceutical composition (for example a pharmaceutical composition for parenteral, oral, topical, mucosal or rectal administration), said process comprising mixing the ingredients.

As mentioned above, such compositions may be prepared e.g. for parenteral, subcutaneous, intramuscular or intravenous administration, particularly in the form of liquid solutions or suspensions; for oral administration, particularly in the form of tablets or capsules; for topical e.g. pulmonary or intranasal administration, particularly in the form of powders, nasal drops or aerosols and transdermal administration; for mucosal administration e.g. to buccal, sublingual or vaginal mucosa, and for rectal administration e.g. in the form of a suppository.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA., (1985). Formulations for parenteral administration may contain as excipients sterile water or saline, alkylene glycols such as propylene glycol, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. Formulations for nasal administration may be solid and may contain excipients, for example, lactose or dextran, or may be aqueous or oily solutions for use in the form of nasal drops or metered sprays. For buccal administration typical excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like.

Compositions suitable for oral administration may comprise one or more physiologically compatible carriers and/or excipients and may be in solid or liquid form. Tablets and capsules may be prepared with binding agents, for example, syrup, acacia, gelatin, sorbitol, tragacanth, or poly-vinylpyrollidone; fillers, such as lactose, sucrose, corn starch, calcium phosphate, sorbitol, or glycine; lubricants, such as magnesium stearate, talc, polyethylene glycol, or silica; and surfactants, such as sodium lauryl sulfate. Liquid compositions may contain conventional additives such as suspending agents, for example sorbitol syrup, methyl cellulose, sugar syrup, gelatin, carboxymethyl-cellulose, or edible fats; emulsifying agents such as lecithin, or acacia; vegetable oils such as almond oil, coconut oil, cod liver oil, or peanut oil; preservatives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Liquid compositions may be encapsulated in, for example, gelatin to provide a unit dosage form.

Solid oral dosage forms include tablets, two-piece hard shell capsules and soft elastic gelatin (SEG) capsules.

A dry shell formulation typically comprises of about 40% to 60% w/w concentration of gelatin, about a 20% to 30% concentration of plasticizer (such as glycerin, sorbitol or propylene glycol) and about a 30% to 40% concentration of water. Other materials such as preservatives, dyes, opacifiers and flavours also may be present. The liquid fill material comprises a solid drug that has been dissolved, solubilized or dispersed (with suspending agents such as beeswax, hydrogenated castor oil or polyethylene glycol 4000) or a liquid drug in vehicles or combinations of vehicles such as mineral oil, vegetable oils, triglycerides, glycols, polyols and surface-active agents.

Pimozide is a well-known dopamine D2 receptor antagonist and its interaction with the dopamine D2 receptor is commonly thought to underlie its neuropharmacological effects. Pimozide is also known to interact with a range of other receptors including the 5-hydroxytryptamine (5-$HT_7$) receptor and hERG. There are therefore advantages associated with formulations of pimozide that deliver the compound to exert an inhibitory effect on RAN at a transcriptional level while avoiding substantial off target effects at, for example, the dopamine receptor. Such formulations advantageously exhibit a selective anti-cancer effect. Accordingly, the present invention provides a nanoparticle delivery system for pimozide that advantageously reduces, for example essentially avoids, any neuropharmacological effect normally associated with pimozide administration.

One established strategy for selective drug delivery involves of encapsulating a drug in a suitable particulate carrier system, thereby allowing the *in vivo* fate of that drug to be determined by the properties of the carrier system rather than its own intrinsic physicochemical and transport properties. As a result, an increased therapeutic index can be achieved through control of the rate and site of drug release as off-target effects and toxicity can be greatly reduced or eliminated. Formulation options for Pimozide thus include the use of particulate carrier systems such as liposomes, microspheres, micelles and nanoparticles.

The use of biodegradable polyester matrices, of various forms, features in many innovative formulation approaches evaluated over the past four decades. For example, goserelin acetate peptide incorporated into PLGA copolymer, and used for treating of advanced prostate and breast cancers, was approved by the FDA in 1998. This delivery system can protect and release the drug slowly into the systemic circulation, where it can reach its target site.

Liposomes and colloidal nanoparticles have been used to encapsulate biologically active drugs with penetration through the cell membrane and intracellular delivery being demonstrated. Polymeric nanoparticles (NP) for example those made from poly(caprolactone) (PCL) and poly(ethylene glycol)/ poly(lactic-co-glycolic acid) (PEG/PLGA) block copolymer

NPs have been found by the present inventors to be especially beneficial for the delivery of pimozide. Accordingly, in one embodiment the invention relates to poly(lactic-co-glycolic acid) (PLGA) comprising nanoparticle formulations of the inhibitors of the invention. The composition of block copolymers provided herein is by percentage weight of the composition excluding the pimozide for use. In one embodiment, PLGA comprising nanoparticle formulations of the inhibitors of the invention are based on PLGA/polyethylene glycol (PEG) block copolymer nanoparticle. In one embodiment the % PEG content of the PLGA/PEG block copolymer nanoparticle is from 1% to 15%, the remainder being PLGA. In one embodiment the % PEG content of the PLGA/PEG block copolymer nanoparticle is from 2.5% to 10%. In one embodiment the % PEG content of the PLGA/PEG block copolymer nanoparticle is between 5% to 10%. Increasing the PEG content advantageously improves the encapsulation efficiency. In one embodiment the invention relates to nanoparticle formulations of pimozide for example, poly(caprolactone) (PCL) comprising nanoparticle (NP) formulations of pimozide. In one embodiment PCL comprising nanoparticle formulations of pimozide are have the following properties/parameters. For PCL-pimozide NP, the nanoparticle formulations generally analysed herein have a mean diameter of 201±26 nm a zeta potential of -28.3 ± 2.24 mV. In one embodiment the nanoparticle formulations have a mean polydispersity index of 0.143±0.017.

The nanoparticle formulations of the invention are generally fabricated using emulsion solvent evaporation or by solvent displacement techniques. In one embodiment the emulsion solvent evaporation technique is used to form nanoparticle formulations of pimozide. The nanoparticle formulations of the invention advantageously enhance the biophysicochemical properties and achieve controlled cytotoxic and apoptotic efficacy on cancer cells relative to free peptide inhibitors while significantly reducing or eliminating the effects of pimozide on the dopamine receptor and/or hERG. The nanoparticle formulations according to the invention have a mean diameter 100 nm to 400 nm, for example from 150 nm to 352 nm. In one embodiment the nanoparticle formulations according to the invention have a mean diameter between 150 and 300nm. In one embodiment the nanoparticle formulations according to the invention have a zeta potential of from -3 to -21 mV mV. In one embodiment the nanoparticle formulations of the invention have a mean polydispersity index of between 0.14 and 0.23. Advantageously, dosing pimozide as a nanoparticle formulation in PCL increases the absorption of pimozide and also significant reduces or essentially eliminates the neuropharmacological effects that result from dosing of the non-NP form. It is believed that phagocytic transport of the nanoparticle formulations of the invention into cells deliver a selective cytotoxic effect on cancer cells. Experiments, such as those described herein, show that treatment of cancer cells in culture with pimozide containing nanoparticles delivers a cytotoxic effect against

cancer cells that is initially (i.e. after 24h) lower than dosing with an equivalent amount of free pimozide but that is equal or greater than that caused by the equivalent amount of free pimozide 96h after administration. This indicates that the dosing with pimozide loaded nanoparticles may provide a sustained release of the drug and this may be therapeutically advantageous as well as avoiding off-target neuropharmacological effects.

**Second active ingredients**

In some embodiments, pimozide, or a pharmaceutically acceptable form thereof, for use or for use in a method of treatment as described herein, can be combined with or comprise one or more second active ingredients which are understood as other therapeutic compounds or pharmaceutically acceptable derivatives thereof.

Methods for treatment according to the present invention in one embodiment thus further comprise one or more steps of administration of one or more second active ingredients, either concomitantly or sequentially, and in any suitable ratios. In one embodiment, such second active ingredients is, for example, selected from compounds used to treat or prevent cancer in the tissue of one or more organs or symptoms or used to treat or prevent complications associated with treatment of cancer in the tissue of one or more organs. In one embodiment, such a second active ingredients is intended for the treatment of the side effects associated with the primary treatment or is aimed at overcoming or suppressing resistance that may arise in the cancer. Thus the one or more second active ingredients may be directed to the treatment of cancer, the treatment or suppression of emesis, the blockage of drug efflux pumps or potentiation of the anti-cancer effect of pimozide.

A preferred class of second active ingredient for use in combination with pimozide are tyrosine kinase inhibitors. The tyrosine kinase inhibitor may be as a small molecule or an antibody tyrosine kinase inhibitor. Examples of tyrosine kinase inhibitors that may be used in combination with a Ran inhibitor, for example pimozide, for the treatment of cancer include afatinib (Giotrif™), axitinib (Inlyta™), bosutinib (Bosulif™), crizotinib (Xalkori™), dasatinib (Sprycel™), erlotinib (Tarceva), gefitinib (Iressa™), osimertinib (Tagrisso™), imatinib (Glivec™), lapatinib (Tyverb™), nilotinib (Tasigna™), pazopanib (Votrient™), regorafenib (Stivarga™), sorafenib (Nexavar™), sunitinib (Sutent™) and ibrutinib (Imbruvica™).

Methods of treatment according to the present invention in one embodiment include a step wherein pimozide is administered simultaneously, sequentially or separately in combination with one or more second active ingredients. In preferred embodiments the action of the second therapeutic agent and pimozide is synergistic.



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**Kit of parts**

The present invention also provides a kit of parts. A kit of parts according to the present invention in one embodiment comprises pimozide or a pharmaceutically acceptable form thereof or a composition thereof as defined herein for treatment of cancer in the tissue of one or more organs in combination with a further active ingredient as described herein. Kits according to the present invention in one embodiment allows for simultaneous, sequential or separate administration of pimozide and one or more second active ingredients as described herein.

In one embodiment the invention provides a package containing pimozide or a pharmaceutically acceptable form thereof and instructions for its use for the treatment of cancer, for example for patients with a cancer that overexpresses RAN or its protein product Ran or Ran mRNA.

**Administration and dosage**

According to the present invention, a composition comprising pimozide or a pharmaceutically acceptable form thereof is in one embodiment administered to a cancer patient having a cancer that overexpresses *RAN* or its protein product Ran in a pharmaceutically effective dose or a therapeutically effective amount.

A therapeutically effective amount of pimozide or a pharmaceutically acceptable form thereof according to the present invention is in one embodiment an amount sufficient to cure, prevent, reduce the risk of, alleviate or partially arrest the clinical manifestations of a cancer that overexpresses RAN or its protein product Ran. The amount that is effective will depend on the severity of the cancer as well as on the weight and general state of the subject. An amount adequate to accomplish this is defined as a "therapeutically effective amount".

In one embodiment of the present invention, the composition is administered in doses of from 1 mg/day to 5000 mg/day.

**Routes of administration**

It will be appreciated that the preferred route of administration will depend on the general condition and age of the subject to be treated, the nature of the condition to be treated, the location of the tissue to be treated in the body and the formulation of the active ingredient chosen. Pimozide may be administered as an oral formulation, for instance in the form of a tablet. If a nanoparticle formulation is to be used, the administration may be in the form a local

infusion or injection, or an intravenous or subcutaneous injection depending on the location of the cancer to be treated.

**Systemic treatment**

In embodiments, the route of administration allows for introducing pimozide, or a pharmaceutically acceptable form thereof, for use into the blood stream to ultimately target the desired site(s) of action.

In embodiments, the route of administration is any suitable route, such as an enteral route (including the oral, rectal, nasal, pulmonary, buccal, sublingual, transdermal, intracisternal and intraperitoneal administration), and/or a parenteral route (including subcutaneous, intramuscular, intrathecal, intravenous and intradermal administration).

Appropriate dosage forms for such administration may be prepared by conventional techniques.

**Parenteral administration**

Parenteral administration is any administration route not being the oral/enteral route whereby the medicament avoids first-pass degradation in the liver. Accordingly, parenteral administration includes any injections and infusions, for example bolus injection or continuous infusion, such as intravenous administration, intramuscular administration or subcutaneous administration. Furthermore, parenteral administration includes inhalations and topical administration.

Accordingly, the peptide or composition is in one embodiment administered topically to cross any mucosal membrane of an animal to which the substance or peptide is to be given, e.g. in the nose, vagina, eye, mouth, genital tract, lungs, gastrointestinal tract, or rectum, for example the mucosa of the nose, or mouth, and accordingly, parenteral administration may also include buccal, sublingual, nasal, rectal, vaginal and intraperitoneal administration as well as pulmonal and bronchial administration by inhalation or installation. In some embodiments, the peptide is administered topically to cross the skin.

In one embodiment, the intravenous, subcutaneous and intramuscular forms of parenteral administration are employed.

In one embodiment, the peptide or composition according to the invention is used as a local treatment, i.e. is introduced directly to the site(s) of action. Accordingly, pimozide may be

applied to the skin or mucosa directly, or may be injected into the site of action, for example into the diseased tissue or to an end artery leading directly to the diseased tissue.

**Alternative pharmaceutical formulations**

In one embodiment, pimozide or a pharmaceutically acceptable form thereof according to the present invention or pharmaceutically acceptable derivatives thereof are administered alone or in combination with pharmaceutically acceptable carriers or excipients, in either single or multiple doses. The pharmaceutical compositions or compounds according to the invention may be formulated with pharmaceutically acceptable carriers or diluents as well as any other known adjuvants and excipients in accordance with conventional techniques such as those disclosed in Remington: The Science and Practice of Pharmacy, 20th Edition, Gennaro, Ed., Mack Publishing Co., Easton, PA, 2000.

The term "pharmaceutically acceptable form" in present context includes pharmaceutically acceptable salts, which indicate a salt which is not harmful to the patient. Such salts include pharmaceutically acceptable basic or acid addition salts as well as pharmaceutically acceptable metal salts, ammonium salts and alkylated ammonium salts. A pharmaceutically acceptable derivative further includes esters and prodrugs, or other precursors of a compound which may be biologically metabolized into the active compound, or crystal forms of a compound.

The pharmaceutical composition or pharmaceutically acceptable composition may be specifically formulated for administration by any suitable route, such as an enteral route, the oral, rectal, nasal, pulmonary, buccal, sublingual, transdermal, intracisternal, intraperitoneal, and parenteral (including subcutaneous, intramuscular, intrathecal, intravenous and intradermal) route.

In an embodiment of the present invention, the pharmaceutical compositions or compounds of the present invention are formulated for crossing the blood-brain-barrier.

Pharmaceutical compositions for oral administration include solid dosage forms such as hard or soft capsules, tablets, troches, dragees, pills, lozenges, powders and granules. Where appropriate, they can be prepared with coatings such as enteric coatings, or they can be formulated so as to provide controlled release of the active ingredient, such as sustained or prolonged release, according to methods well known in the art. In the same solid dosage form two active ingredients may be combined so as to provide controlled release of one active ingredient and immediate release of another active ingredient.

Liquid dosage forms for oral administration include solutions, emulsions, aqueous or oily suspensions, syrups and elixirs.

Pharmaceutical compositions for parenteral administration include sterile aqueous and non-aqueous injectable solutions, dispersions, suspensions or emulsions, as well as sterile powders to be reconstituted in sterile injectable solutions or dispersions prior to use. Depot injectable formulations are also regarded as being within the scope of the present invention.

Other suitable administration forms include suppositories, sprays, ointments, creams/lotions, gels, inhalants, dermal patches, implants, etc.

In one embodiment, pimozide for use according to the present invention is generally utilized as the free substance or as a pharmaceutically derivative such as a pharmaceutically acceptable salt thereof. Examples of the latter are an acid addition salt of pimozide or a pharmaceutically acceptable form thereof. The term "pharmaceutically acceptable salt" refers to a nontoxic salt of a compound for use according to the present invention, which salts are generally prepared by reacting a free base with a suitable organic or inorganic acid. Salts of pimozide derivatives containing a free base functionality can be prepared in a conventional manner by treating a solution or suspension of the compound with a chemical equivalent of a pharmaceutically acceptable acid. Pharmaceutically acceptable acid addition salts include, but are not limited to, hydrochloride, hydrobromide, hydroiodide, nitrate, sulfate, bisulfate, phosphate, acid phosphate, isonicotinate, acetate, lactate, salicylate, citrate, tartrate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzensulfonate, p-toluenesulfonate and pamoate (i.e., 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts.

The term "prodrug" refers to derivatives of pimozide that are rapidly transformed *in vivo* to yield the pimozide, for example, by hydrolysis in blood or by metabolism in cells, such as for example the cells of the basal ganglia. A thorough discussion is provided in T. Higuchi and V Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are hereby incorporated by reference. Examples of prodrugs include pharmaceutically acceptable, non-toxic esters of the compounds of the present invention.

Suitable pharmaceutical carriers include inert solid diluents or fillers, sterile aqueous solutions and various organic solvents. Examples of solid carriers are lactose, terra alba, sucrose,

cyclodextrin, talc, gelatine, agar, pectin, acacia, magnesium stearate, stearic acid and lower alkyl ethers of cellulose. Examples of liquid carriers are syrup, peanut oil, olive oil, phospholipids, fatty acids, fatty acid amines, polyoxyethylene and water. Moreover, the carrier or diluent may include any sustained release material known in the art, such as glyceryl monostearate or glyceryl distearate, alone or mixed with a wax. The pharmaceutical compositions formed by combining the compounds for use according to the present invention and the pharmaceutically acceptable carriers are then readily administered in a variety of dosage forms suitable for the disclosed routes of administration. The formulations may conveniently be presented in unit dosage form by methods known in the art of pharmacy.

Formulations of the present invention suitable for oral administration may be presented as discrete units, such as capsules or tablets, which each contain a predetermined amount of the active ingredient, and which may include a suitable excipient.

Furthermore, the orally available formulations may be in the form of a powder or granules, a solution or suspension in an aqueous or non-aqueous liquid, or an oil-in-water or water-in-oil liquid emulsion.

Compositions intended for oral use may be prepared according to any known method, and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavouring agents, colouring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets may contain the active ingredient(s) in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets. These excipients may, for example, be: inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example corn starch or alginic acid; binding agents, for example, starch, gelatine or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. They may also be coated by the techniques described in U.S. Patent Nos. 4,356,108; 4,166,452; and 4,265,874, the contents of which are incorporated herein by reference, to form osmotic therapeutic tablets for controlled release.

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as a liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard

paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and flavouring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active compound in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example, sweetening, flavouring, and colouring agents may also be present.

Syrups and elixirs may be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative and flavouring and colouring agent. The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known methods using suitable dispersing or wetting agents and suspending agents described above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conveniently employed as solvent or suspending medium. For this purpose, any bland fixed oil may be employed using synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The compositions may also be in the form of suppositories for rectal administration of the compounds of the invention. These compositions can be prepared by mixing the compound with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal temperature and will thus melt in the rectum to release the drug. Such materials include, for example, cocoa butter and polyethylene glycols.

**Examples**

The present invention is illustrated by the following examples, which are not intended to limit the scope of the invention.

**Pimozide treatment causes a dose dependent reduction in Ran mRNA and Ran protein**

To establish the effects of Pimozide treatment on Ran mRNA and protein levels in cancer cells quantitative Real Time PCR (RT-PCR) experiments and Western blots were performed. In more detail, MDA MB 231 breast cancer cells (Figures 1A and 1C) and A549 (Figure 1B) lung

cancer cells were either untreated (control) or treated with 5 μM to 15 μM concentrations of pimozide. RNA was extracted from the cells after 48h and quantitative RT-PCR was then performed with a ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The Taqman probe sets for RAN and 18s RNA (assay id 319913E-0702028) were used. Gene expression was normalized to 18s RNA. As can be seen a dose dependent decrease in Ran mRNA in treated cells was observed indicating that pimozide decreases the transcriptional activity of *RAN*.

To establish the effects of pimozide treatment on Ran down stream target genes mRNA levels in cancer cells quantitative Real Time PCR (RT-PCR) experiments were performed. In more detail, MDA MB 231 breast cancer cells (Figure 1D), were either untreated (control) or treated with 5 μM to 20 μM concentrations of pimozide. RNA was extracted from the cells after 48h and quantitative RT-PCR was then performed with a ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The Taqman probe sets for *RAN* and its target genes and 18s RNA were used. Gene expression was normalized to 18s RNA. As can be seen a dose dependent decrease in Ran, C-MET and MMP2 mRNA in treated cells was observed indicating that pimozide decreases the transcriptional activity of *RAN* down stream targets.

**Pimozide inhibits *RAN* at a transcriptional level**

To further probe the effect of Pimozide treatment on *RAN* expressing tumour cells a dual luciferase reporter assay was developed based on a pGL3 basic reporter construct (Promega, Southampton, UK) into which full length Ran promoter (1400 base pairs upstream of the transcriptional start site, amplified using Phusion Hot Start High-Fidelity DNA polymerase) and partial sequences thereof (770bp, 368 bp and 162 bp segments from the N-terminal region derived by serial deletion) were incorporated. The resultant constructs along with the unmodified construct were then transfected into MDA MB 231 cancer cells. Cells containing the various constructs were then treated with or without pimozide for 24 hours. The firefly luciferase activity was normalized to the internal control Renilla luciferase activity.

Results from the dual luciferase assay are presented in Figure 2A. In cells in which the unmodified pGL3 construct was incorporated, the luciferase activity in control and pimozide treated cells was equivalent, i.e. pimozide treatment had no effect. In contrast, luciferase activity in cells with the pGL3 full length Ran promoter construct and 700bp construct showed essentially no luciferase activity after pimozide treatment indicating that pimozide treatment directly inhibits *RAN* at a transcriptional level. The constructs with shorter, 368bp and 162bp

fragments of the Ran promoter construct, had little or no luciferase activity irrespective of pimozide treatment status.

It is thus evident that the transcription factor that is downregulated by pimozide is present in the 700 bp fragment of the Ran promoter but is not in either 368 bp or 162 bp fragments. Myc has been identified as a transcription factor that regulates Ran in the level of transcription (Figure 2B, data from MDA MB-231 cells)

Figure 3 shows the results from Western blots of Ran GTPase activity in MDA MB 231 cell in untreated cells and cells treated with 10 μM and 15 μM of pimozide. In more detail, Ran GTPase activity was measured using the Ran activation assay kit according to the manufacturer's instructions (Cell Biolabs, San Diego, CA). Briefly, GTP-bound Ran was pulled down by using RanBP1 PBD Agarose bead slurry, and the pulled-down complex was then dissociated by boiling in 2× reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer. The pull-down supernatant was then analyzed by Western blotting. As can be seen pimozide treatment reduces the amount of RanGTP protein compared to the control (DMSO) treated cells.

A related immunoprecipitation experiment was performed to assess whether RCC1 expression was down regulated along with Ran. This experiment demonstrated pimozide treatment had no effect on RCC1 expression (data not shown).

**Pimozide selectively inhibits the growth of cancer cells relative to non-cancerous cell**

The relative effect of pimozide treatment on normal mammary epithelial cells and cancer cells is shown in Figure 4 MDA-MB 231 human breast cancer cells, A549 human lung cancer cells and MCF10A mammary epithelial cells were incubated with pimozide for 48 hours. A 12.5 μM concentration caused a ca 50% reduction in cell viability in the cancer cells while the normal mammary epithelial cells were relatively unaffected demonstrating the selective cytotoxicity of pimozide to the *RAN* expressing cancer cell lines.

The effect of pimozide treatment on the cell cycle in MDA MB231 and A549 cells 24 and 48 hours post treatment was also evaluated. Both cancer cell lines showed an increased apoptosis as drug does increased as shown by sub-G1 cell populations at 48 hours, pimozide increased cell apoptosis by ~30% with 10 μM. Pimozide was observed to provoke DNA damage response (DDR) in MDA MB-231 breast cancer cells by increasing DNA lesions and fragmentations (red arrow) with chromatin condensation (yellow ahead arrow) as results of drug treatment, there were presence of DNA blebbing after a 48 hour exposure to 7.5 μM pimozide (white arrow) (Figure 4Bi = control, Figure 4Biv = pimozide treated cells). Pimozide

also caused double-strand DNA breaks (DSBs) by increasing expression of phosphorylated H2A histone family, member X (γ-H2AX) with effect higher than the FDA-approved anti-cancer drugs, Doxorubucin and Paclitaxel (Figure 4Biii = control, Figure 4Biv = 48 hour exposure to 7.5 μM pimozide).

**Pimozide reduces the tumour burden in a nude mice xenograft model, decreases cell proliferation and metastasis incidence in lung.**

Female C57/BL6 nude mice (8-weeks old), kept and handled according to institutional guidelines, complying with UK legislation under 12/12 hours light/dark cycle at a temperature of 22°C, received a standard diet and acidified water ad libitum. MDA-MB-231-Luc cells (2 × 106) were injected subcutaneously in 100 μL phosphate-buffered saline together with 100 μL Matrigel basement membrane matrix (Becton Dickinson) into one fat pad of each mouse.

Mice were randomly assigned to cohorts of ten mice each, and were grouped in four groups. Group-1 (G1), a tumour free control group, were injected with matrigel without MDA MB 231 cell and were not treated with pimozide. Group-2, a tumour bearing control group, received MDA MB 231 cells in matrigel and were not treated with pimozide but were instead injected with an equal volume of vehicle (PBS) in tandem with treatment in group-4 (G2NT); Group-3, the early treatment cohort, received MDA MB 231 cells in matrigel and was treated after 24 hours after implantation with 20 mg/kg i.p. of pimozide once daily in a five on, 2 off dosing regime (once daily dosing from Monday to Friday with no dosing on Saturday or Sunday) for the duration of the study. Group-4, the long term dosing cohort, were injected with cells and the tumours were left for 14 days to become established (G4TL). From day 14 onwards, the group Group-4 cohort were treated by i.p. injection with 20mg/kg pimozide in a once daily 5 on, 2 off dosing regime (once daily dosing from Monday to Friday with no dosing on Saturday or Sunday).

Throughout the study the shortest and longest diameter of the tumours were measured with calipers at the indicated time intervals, and tumour volume ($mm^3$) was calculated using the formula: $(\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$. Animal body weight and any sign of morbidity were monitored. Drug treatment lasted 4 weeks. Animals were euthanized 24 hours after the last drug administration according to institutional guidelines, and then tumours were carefully removed, weighed and analysed. A total necropsy analysis involving tumours and distinct organs was carried out.

Tumour tissue samples obtained at the end of the study were fixed in 4% (w/v) buffered paraformaldehyde and embedded in paraffin. Tissue sections (4-5 μm) were deparaffinized and hydrated in graded ethanol and distilled water for analysis. Endogenous peroxidase

activity was blocked using methanol and 30% $H_2O_2$ for 30 minutes. Sections were counterstained with hematoxylin and eosin (H&E, Figure 6 row 1), and then incubated overnight at 4ºC with different antibodies: rabbit anti-human Ki67 monoclonal antibody (clone SP6) (1:1000 dilution, Abcam, Figure 6 row 2), anti-active human cleaved-caspase-3 (1:200 dilution for IHC & 1:100 for IF, Cell signaling, Figure 6 row 3) rabbit polyclonal antibody that specifically recognized the active ~20-kDa subunit (Cell signaling) anti-human Ran (1:1000, Millipore, Figure 6 row 4), anti-human MMP2 (1:100 dilution, Millipore), anti-Akt1, anti-Akt2, pAkt (1:1000, Cell Signaling) and anti-human CD31 (1:50 for IHC and 1:20 for IF, Abcam) and α-smooth muscle actin (α-SMA)(1:50 for IHC and 1:100 for IF) (Abcam).b After washing with PBS, sections were incubated with biotinylated anti-rabbit IgG antibody (BD Pharmingen) for 60 minutes at room temperature and washed with PBS. Then, sections were incubated with streptavidin horseradish-peroxidase (Vector Laboratories) for 60 minutes in a moist chamber, washed with PBS, and sites of peroxidase activity were visualized using 3,3′-diaminobenzidine tetrahydrochloride solution (DAB). Sections were subsequently counterstained with hematoxylin Mayer's & Eosin (Sigma). Staining was analyzed with a Nikon Eclipse 400® microscope and Metamorph® software (Molecular Devices Corporation).

Images of the tumours at the end of the study are shown in Figure 5A and plots of tumour volume at the end of treatment and metastasis numbers are provided in Figures 5B and 5C, respectively. As can be seen from Figure 5B tumour volumes were reduced by 65% ($p<0.05$) (Group-3) and by 60% ($p<0.05$) (Group-4). In addition, tumor incidence in treated groups were delayed by 55% ($P<0.05$) (Group-3) (data not shown).

The lungs of the mice from the treated and untreated groups at the end of the study were checked for metastases. Mice in the untreated cohort showed high degree of metastasis in lung compared to treated animals. Very few focal metastasis were observed in airway epithelium in the treatment group. The level of Ran protein expression in lung metastasis foci was studied by immunohistochemistry (see Fig 6, row 4). This revealed a higher expression of Ran in the lung metastases present in the untreated cohort and lower Ran expression in the lung metastases present in each, early and late, treated tumour cohort. Counting of the metastases in the treated/untreated cohorts showed a significant reduction in metastases in the treated cohorts relative to untreated control (see Figure 5C).

Immuno-staining of tumour tissue samples with Ki67, a cell proliferation marker, revealed a decrease in cell proliferation in both treated cohorts (Groups 3 (early treatment TE) and 4 (late treatment TL)) compared to the untreated tumour bearing cohort (Group-2) (Figure 6, row 2).

Staining of the tumour tissue for cleaved-caspase-3 is shown in Figure 6, row 3. Caspase 3 activation was seen to be increased in Group 3 and Group 4 treated tumours (columns 2 and 3 respectively) compared to untreated tumours (column 1) which indicates an increase of apoptosis index at the cellular level *in vivo* (indicated by arrows).

Staining of the tumour tissue samples for Ran protein indicated that Ran protein levels were reduced in treated tumours in TE and TL groups (Group 3 and Group 4 respectively, Figure 6, row 4) relative to the untreated cohort.

**Effect of Pimozide on cell migration, invasion and metalloproteinase MMPs expression.**

Ran protein is known to have a role in carcinogenesis and metastasis. Pimozide treatment was thus evaluated for effects on motility characteristics of different human breast cancer cell lines *in vitro*. Migration experiments using MDA-MB-231 and MCF7 cell lines in the presence of 7.5 µM pimozide were performed. A wound healing scratch closure assay was performed to determine changes in cellular motility. For this assay, $1x10^5$ MCF7 or $6x10^4$ MDA-MB-231 human breast cancer cells were seeded into each well of 24-well tissue culture plates and left to grow for 48 hours at which stage they reached 90-95% confluency. The resultant monolayers were then scored with a sterile pipette tip prior, for the treatment cohorts, to addition of 7.5 µM pimozide in normal medium. Wound closure was monitored by collecting digitalized images using the Cell-IQ automated image capture system, (Chip-Man Technologies) on preselected fields using phase contrast microscopy. Analysis of the collected data was performed using the Cell-IQ Analyser Software, to generate percentage of wound closure over time. Quantification of the closed area at the 24 hours' time point were singled out for all conditions and normalized as percentage to the control untreated cells.

It was observed that whilst untreated cells underwent significant collective migration into the empty space 24 hours post scratching, pimozide treated cells demonstrated clear defects in their abilities to migrate. Quantification of the cells ability to close the gap further demonstrated a reduction by 40% ($P<0.05$) of the wound area in both MDA-MD-231 and MCF7 breast cells in the presence of pimozide.

Relative expression of MMP1 and MMP14 at mRNA level was also decreased in MDA-MB-231 cells treated with pimozide at 7.5 µM after 24 hours compared to control. This indicates that pimozide exerts a negative effect on metalloproteinase activation (i.e. reduces activation of MMPs) by reducing Ran protein expression.

**Pimozide inhibits migration capacities of both MDA-MB-231 and A549 cancer cells.**

To provide further evidence of the migratory deficiencies and possibly provide clues about the molecular mechanisms entailed, MDA-MB-231 and A549 were stained for different markers of motility (Figure 7). Paxillin is a component of the focal adhesion, acting as an adaptor protein between integrins and the actin cytoskeleton. Myosin IIA is usually found in the lamella of motile cells, just behind the lamellipodium as well as throughout the cell body, being mainly responsible for the contraction forces require during the organization of actin stress fibers; whilst Arp3 is a component of the Arp2/3 complex that promote actin nucleation and polymerization, mainly located at the leading edge and principally the lamellipodium of migratory cells. Staining MDA-MB-231 cells for all these markers demonstrated their motile and invasive nature, with a clear polarization of these cells, demonstrating a well-defined leading edge (Figure 7A). Lamellipodia and filopodia structures were also clearly visible; along with little actin stress fibers throughout the cell bodies. Treatment of cancer cells with 7.5μM pimozide resulted in dramatic changes in the overall cytoskeletal architectures of these cells. Polarization and the presence of a leading edge was lost in most cancer cells, along with a dramatic reduction in the numbers of cellular protrusion, reduced from an average of 65±10 per cells in the control cells to around 15± 8 in the treated counterparts. A severe concentration of both actin stress fibers and punctate actin clusters could be seen in the body of the treated cells (Figure 47 actin staining). Whilst the presence of focal adhesion was minimal in the control untreated cells, an indication of dynamic reorganization of these protein complexes and a sign of motility, incubating the cells with pimozide resulted in the presence of paxillin foci at localized regions of the cell body (Figure 7 paxillin staining).

**Pimozide affects epithelial mesenchymal transition (EMT) markers in MDA-MB-231 cells**

A RT-PCR-based analysis of two EMT markers (Vimentin and Zo-1) in MDA-MB-231 breast cancer cell lines treated with pimozide during 24 hours revealed a significant decrease of mRNA expression with more than 90% in both markers at $p<0.05$ (Figure 8A). The relative gene expression of EMT markers was also checked at mRNA level and revealed a decrease of relative gene expression of most EMT markers (Ncadherin, Vimentin, Snail, Slug and Twist) (Figure 8B) except E-cadherin that was maintained during the treatment (data not shown). Furthermore, treatment of MDA-MB-231 with pimozide also caused a decrease in Snail, Vimentin and N-cadherin protein expression as seen in the Western blot (Figure 8C). To further validate this, a functional *in vivo* assay of mice bearing tumours from invasive breast cancer treated earlier (Group-3) or late (Group-4), showed, by immunohistochemistry, a significant decrease a protein expression of vimentin in all mouse tumour samples (Figure 8D).

The Epithelial Mesenchymal Transition (EMT) has an important role in embryonic development and it has also recently been implicated in tumour invasiveness. Acquisition of the EMT phenotype is believed to be central to a cell's ability to metastasize to distant sites, therefore enhancing tumour progression. In this experiment it was found that pimozide significantly inhibits MDA-MB-231 cell growth, migration, invasion and tumourigenicity *in vivo* via suppression of the EMT, a process involving the breakdown of cell-cell junctions and loss of epithelial polarity. Twist and Snail are transcription factors that regulate the expression E-cadherin and N-cadherin, breast cancer patients with high expression of Twist and Snail have poorer progression-free survival and overall survival, suggesting that cadherin expression impacts survival 45,46. In our study, N-cadherin, vimentin and transcription factors Twist, Slug, and Snail expression were reduced both in vitro and in vivo untreated tumours.

Based on these results pimozide appears to have good potential for use in the treatment of tumours with a high metastatic potential, for example as an inhibitor of the Epithelial Mesenchymal Transition, for example in a human patient that has been diagnosed as having a cancer, in particular a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA.

**A polymer nanoparticle formulation of Pimozide can be used to deliver to increase drug absorption and bypass the dopamine receptor: Preparation of pimozide loaded nanoparticles (NP)**

As pimozide is relatively hydrophobic, two different techniques could be employed for forming pimozide loaded nanoparticle formulations. A modified single emulsion solvent evaporation approach or a modified nanoprecipitation technique could be used. Pimozide (0.5 mg) was mixed with 5% w/v of either PCL or PEG-PLGA block copolymer and dissolved overnight in 2 mL of dichloromethane (DCM) or acetone. The resultant organic phase was injected by syringe into 50 ml of a 1.25% aqueous solution of PVA (polyvinyl alcohol) under agitation followed by homogenisation using a Silverson L5T Homogeniser (Silverson Machines, UK) at 10,000 rpm for 7 minutes in case of solvent evaporation technique. The emulsion was stirred by magnetic agitation overnight under vacuum to evaporate the dichloromethane completely and prevent pore formation on the surface of the nanospheres. After the nanospheres had formed, they were centrifuged at 10,000 rpm for 30 min at 4 °C then washed three times with distilled water and 2% w/v sucrose solution and finally freeze-dried. The final product was stored in a desiccator at room temperature.

**Cellular uptake of NP formulations**

Cellular uptake of coumarin 6-loaded NP formulations was evaluated using flow cytometry and fluorescence microscopy [J Pharm Pharmacol 64 (2012) 61-67]. For FACS analysis, coumarin 6 tagged NPs were suspended in Optimem® media and added to MDA-MB-231 cells for 24 hours in 6-well plates. Cells were removed by trypsinisation and resuspended in FACS buffer. Cellular uptake of NP formulations was quantified by gating for positive couramin 6 staining in the FITC channel following control staining with coumarin 6-treated and unstained MDA-MB-231 cells. Three independent experiments with three replicates were performed for each assay.

Cellular uptake and cellular localisation of the pimozide-loaded NP was evaluated qualitatively by fluorescence microscopy by analysis of five fields per well. Intracellular uptake of coumarin 6-loaded NP into MDA-MB-231 cells was detected using fluorescence imaging 24 hours after treatment (Olympus IX70). The images were captured using digital photography (Olympus DP-71, Olympus, Center Valley, PA, USA). MDA-MB-231 cells where seeded into a 6-well plate, containing two fixed cover slips and 2.0 ml of growth medium. After washing the cells with sterile PBS, the coverslips were removed, mounted with fixing media over a glass slide and examined. The untreated MDA-MB-231 cells and cells treated with a solution of coumarin 6 were used as positive and negative controls. As can be seen in Figure 11, over 80% uptake of pimozide loaded in 5% PEG PLGA nanoparticles was observed. Delivery of PCL nanoparticle in contrast was less than 20%.

As can be seen from Figure 10 treatment with pimozide loaded 5% PEG-PLGA NP caused a significant increase in fluorescence shift in the cells (fluorescence peak compared to the control and dye peak). A relatively weak fluorescence shift was seen as a result from treatment with the corresponding dose of PCL NP relative to the control and Coumarin-6 loaded control. (This figure is for cellular uptake results).

**Cytotoxicity studies on NP formulations**

Cytotoxicity of pimozide NP formulations at 1, 2.5, 5 and 10 μM was measured by assessing cell viability (MTT assay). MDA-MB-231 cells were seeded in 24-well plates (Nalge Nunc International, Rochester, NY) at a density of 5 x $10^4$ cells per well and incubated for 24 hours to allow for 60-70% confluency and sufficient adhesion. The cells were treated with different concentrations of the pimozide-loaded NP, pimozide and blank NP. After 24, 48, 72 and 96 hours, the treated cells were washed with 500 μl PBS, and 500 μl of 15% MTT dye solution in complete media added to each well. The plates were incubated at 37 °C and 5% $CO_2$ for an additional 3 hours. The supernatant was then discarded, 500 μl DMSO added and the solution

vigorously mixed. The optical density of each well was measured at 570 nm (reference wavelength 630 nm) in a microplate reader (Fluostar Omega, BMG Lab Tech GMBH, Germany). This experiment was performed in triplicate and repeated three times. Mean values ± SD for each concentration was determined. Percentage cell viability was determined as the ratio of absorbance (570 nm) in treated cells relative to the absorbance in control cells (570 nm). The absorbance of the untreated cells was set at 100%.

The results of this study are shown in Figure 9 and indicate that the 5%-PEG-PLGA NP formulations were at least equipotent to the corresponding doses of free pimozide after 72h and 96h.

**Activity of Pimozide and Gefitinib, as single agents or in combination, against the survival of HCC827 human lung cancer cells and HCC827 GR5 (gefitinib resistant) human lung cancer cells**

As can be seen in Figure 12a, after 24h of treatment with the epidermal growth factor receptor (EGFR) inhibitor gefitinib at all of the evaluated concentrations above 15 nM the survival of the HCC827 cells ranges from *ca* 50 to 60 %. Treatment with 5 μM pimozide causes a reduction in cell survival relative to control of approximately 40%. Treatment of HCC827 cells with gefitinib (at 300 nM) with a 5 μM concentrations of pimozide causes a reduction in cell survival of *ca* 80%, thus illustrating a synergistic effect against cancer cell survival that is realised from the combination of a Ran inhibitor and a EGFR inhibitor (gefitinib).

Figure 13 shows the effects of drug treatment for 24h on the survival of gefitinib resistant HCC827 GR5 cells, a cell line that is derived from normal HCC827 human lung cancer cells (*cf* Figure 12). As can be seen in Figure 13A, treatment with gefitinib in this instance at all of the concentrations evaluated causes no more than a 15% reduction in cell survival. Pimozide treatment (Figure 13B) causes a ca 30% reduction in cell survival at a concentration of 5 μM. The combination of various concentrations of pimozide and gefitinib (300 nM) meanwhile proved superior to either agent when they were presented as single agents. As can be seen in Figure 13C, a *ca* 40% reduction in cell survival at a pimozide concentration was observed at 5 μM pimozide, while higher concentrations of pimozide saw cell survival fall to 20% at 7.5 μM and to less than 10% at 12 μM. It is thus evident that pimozide restores sensitivity to the EGFR inhibitor gefitinib in these resistant cells. Furthermore, as with the data obtained in the wild type HCC827 as presented in Figure 12, the combination of the EGFR gefitinib inhibitor (gefitinib) and the Ran inhibitor (pimozide) proves to be synergistic since the reduction in cell survival observed is greater than additive.

Parallel studies with other Ran inhibitors and tyrosine kinase inhibitors, including inhibitors of EGFR and Bcr-Abl, carried out by the present inventors have shown that Ran inhibitors can act in a synergistic manner with tyrosine kinase inhibitors thus enhancing the anticancer effect of tyrosine kinase inhibitors.

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## CLAIMS

1) Pimozide, or a pharmaceutically acceptable form thereof, for use as an inhibitor of *RAN* at a transcriptional level.

2) Pimozide for use according to claim 1, for use in the treatment of a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA.

3) Pimozide for use according to claim 1 or claim 2, wherein the use is in a patient identified as having a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA.

4) Pimozide for use according to claim 3, wherein the patient identified as having a cancer that overexpresses *RAN*, or its protein product Ran or Ran mRNA, is identified by analysis of a tumour biopsy or a blood sample obtained from that patient.

5) Pimozide for use according to any preceding claim, wherein the patient is a human patient.

6) Pimozide for use according to any preceding claim wherein the pimozide for use is administered in a dosage of 1 to 5000 mg/day.

7) Pimozide for use according to any preceding claim, wherein the pimozide for use is in the form of a nanoparticle formulation.

8) Pimozide for use according to claim 7, wherein the nanoparticle formulation comprises a poly(lactic-co-glycolic acid) copolymer, for example a poly(lactic-co-glycolic acid) / polyethylene glycol block copolymer.

9) Pimozide for use according to any preceding claim in combination with a tyrosine kinase inhibitor.

10) Pimozide for use according to claim 9, wherein the tyrosine kinase inhibitor is an inhibitor of EGFR.

11) Pimozide for use according to claim 9 or claim 10, wherein the pimozide for use and the tyrosine kinase inhibitor is administered simultaneously, sequentially or separately.

12) Pimozide for use according to any of claims 9 to 11, wherein the tyrosine kinase inhibitor is gefitinib.

13) A nanoparticle formulation of pimozide or a pharmaceutically acceptable form thereof.



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14) Formulation according to claim 13, wherein the nanoparticle formulation comprises a poly(lactic-co-glycolic acid) / polyethylene glycol block copolymer.

15) Formulation according to claim 14, wherein the block copolymer comprises from 2.5% to 10% by weight of polyethylene glycol.

16) A method of treatment of a cancer that overexpresses *RAN*, or its protein product Ran or Ran mRNA, comprising a step of administering to a patient in need thereof an effective amount of pimozide or a pharmaceutically acceptable form thereof.

17) The method of claim 16, further comprising the step of testing a sample obtained from said patient to determine whether said patient has a *RAN* overexpressing cancer prior to said administration step.

18) A method of treatment of a cancer that overexpresses *RAN*, or its protein product Ran or Ran mRNA, comprising the step of administering to a patient who has been identified as having a cancer that overexpresses *RAN*, or its protein product Ran or Ran mRNA, an effective amount of pimozide, or a pharmaceutically acceptable form thereof.

19) The method of claim 18, further comprising the step of testing a sample obtained from said patient to determine whether said patient has a *RAN* or Ran or Ran mRNA overexpressing cancer prior to said administration step.

20) A method of treatment of a cancer that overexpresses *RAN*, or its protein product Ran or Ran mRNA, comprising the steps of selecting a patient who has a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA, and administering an effective amount of pimozide or a pharmaceutically acceptable form thereof to that patient.

21) The method of claim 16, further comprising the step of testing a sample obtained from said patient to determine whether said patient has a *RAN* overexpressing cancer prior to said administration step.

22) A method of treatment for cancer in a patient diagnosed as having a cancer characterised by an overexpression of *RAN*, or its protein product Ran or Ran mRNA, involving administration to a patient in need thereof an effective amount of pimozide.

23) Use of pimozide, or a pharmaceutically acceptable form thereof, for the manufacture of a medicament for the treatment of cancer, optionally wherein the cancer overexpresses *RAN* or its protein product Ran or Ran mRNA.

24) Use according to claim 23, wherein the medicament is in a package with instruction for use in the treatment of a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA.

25) Package comprising pimozide or a pharmaceutically acceptable form thereof and instructions for its use in the treatment of a cancer that overexpresses *RAN* or its protein product Ran or Ran mRNA

26) Package according to claim 25, additionally comprising a tyrosine kinase inhibitor.

27) Package according to claim 26, wherein the tyrosine kinase inhibitor is an inhibitor of EGFR, for example gefitinib.

Figure 1A

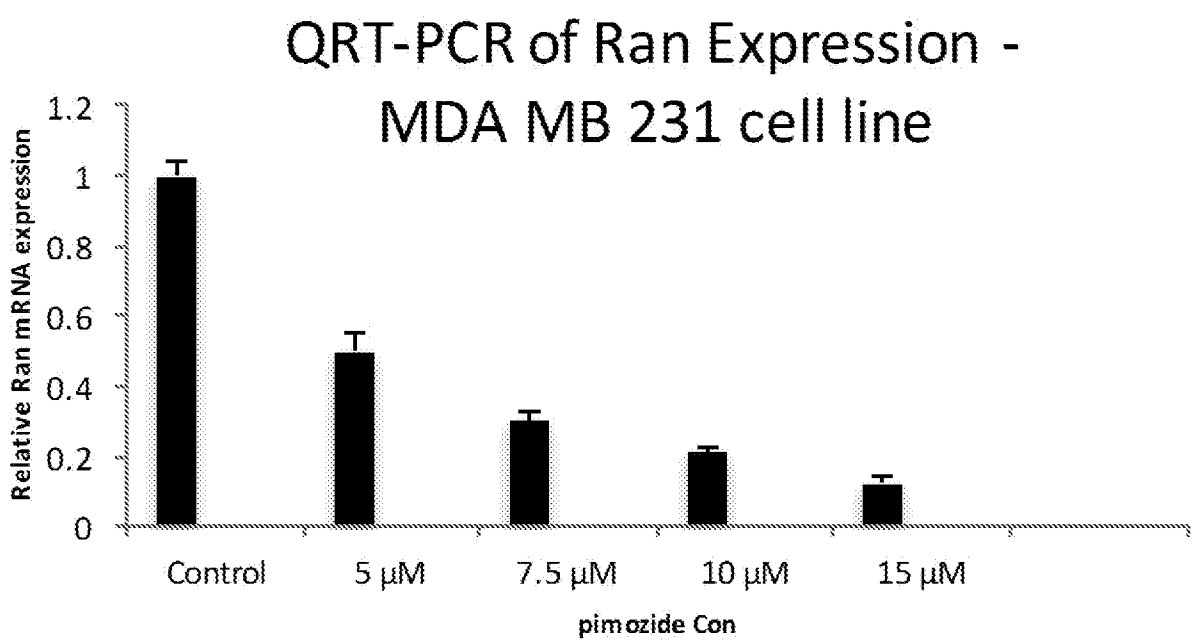



Figure 1B

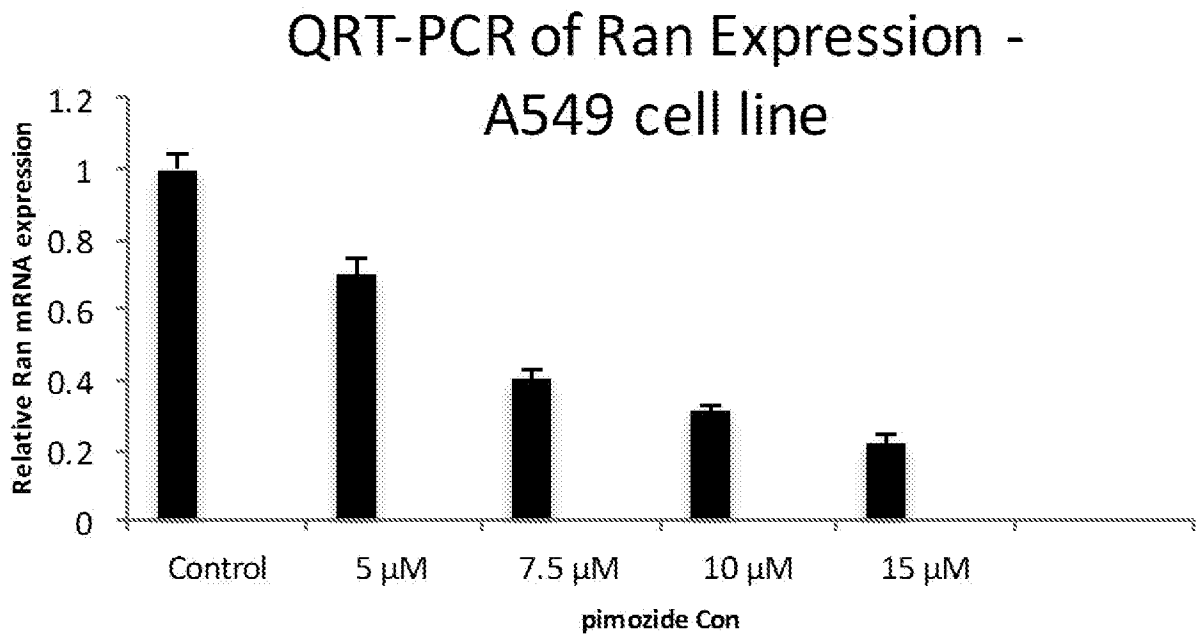


**Figure 1C**

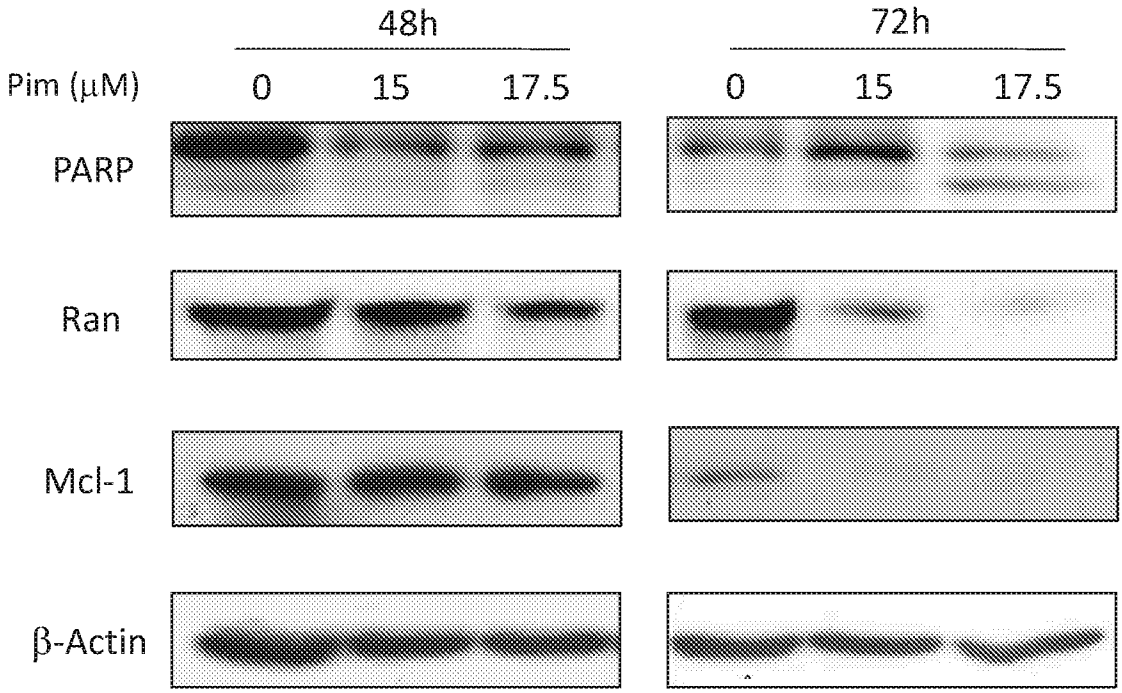
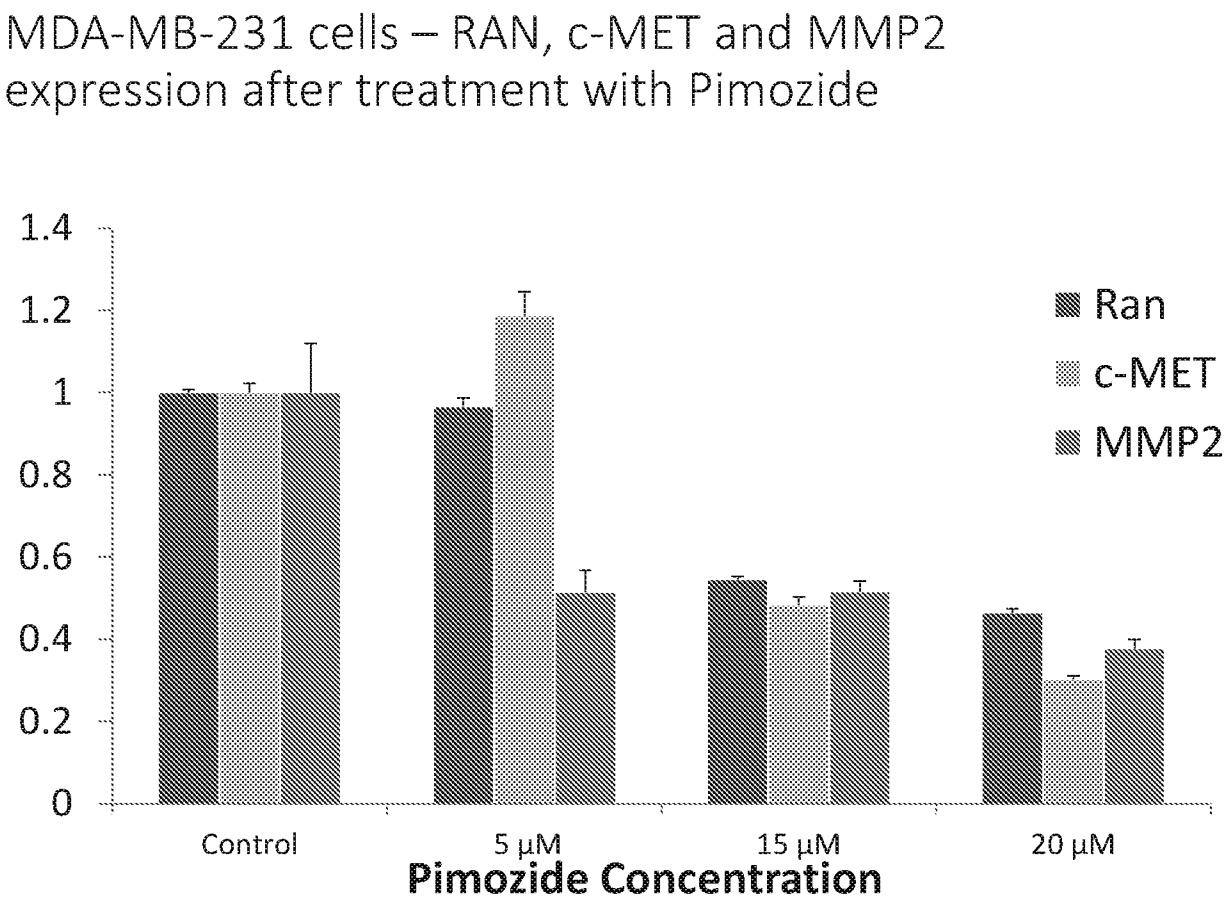
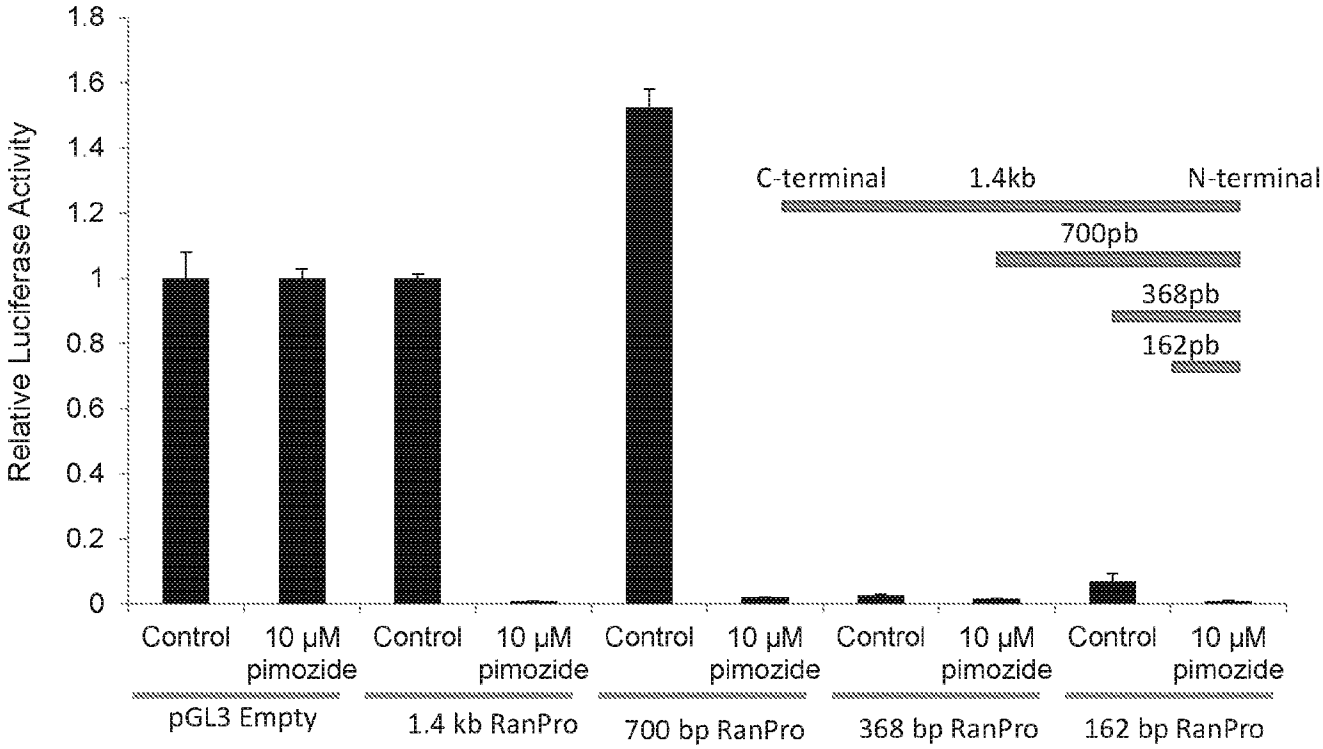



**Figure 1D**

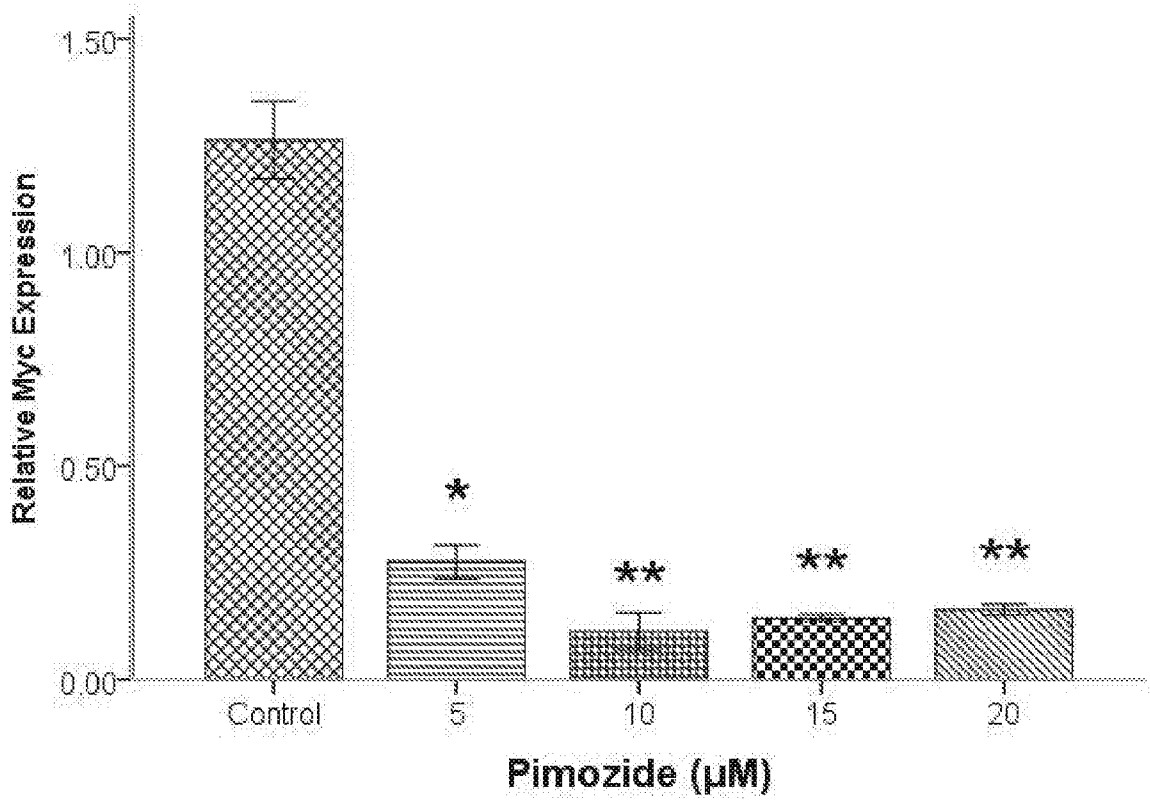


## Figure 2A






## Figure 2B




* p<0.05, ** p<0.01 vs DMSO Control

Figure 3

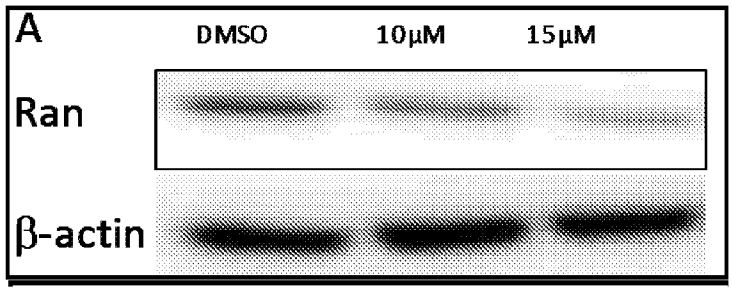



Figure 4A

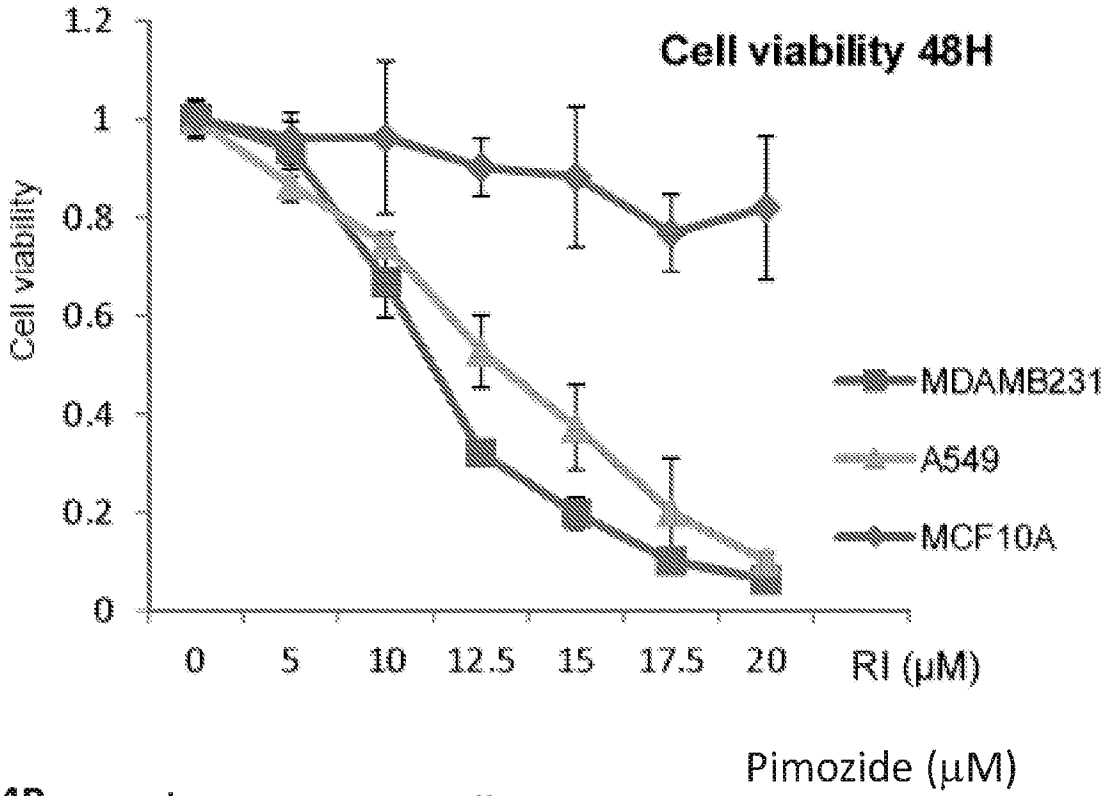



Figure 4B

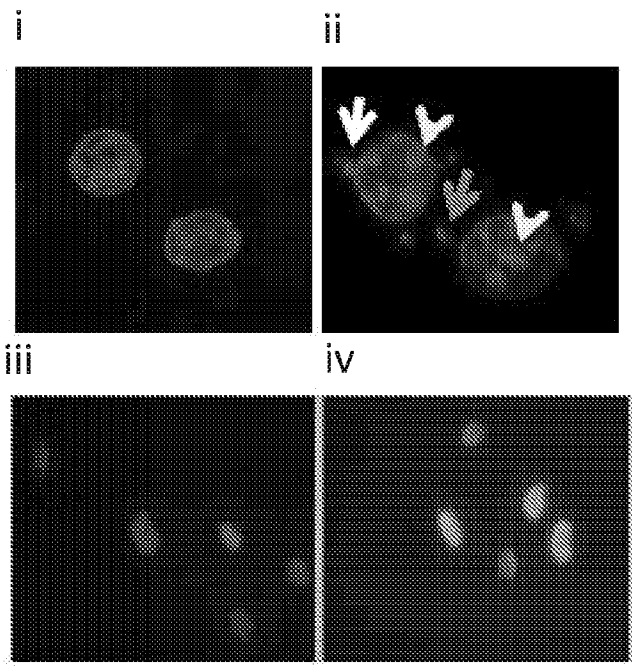


Figure 5A

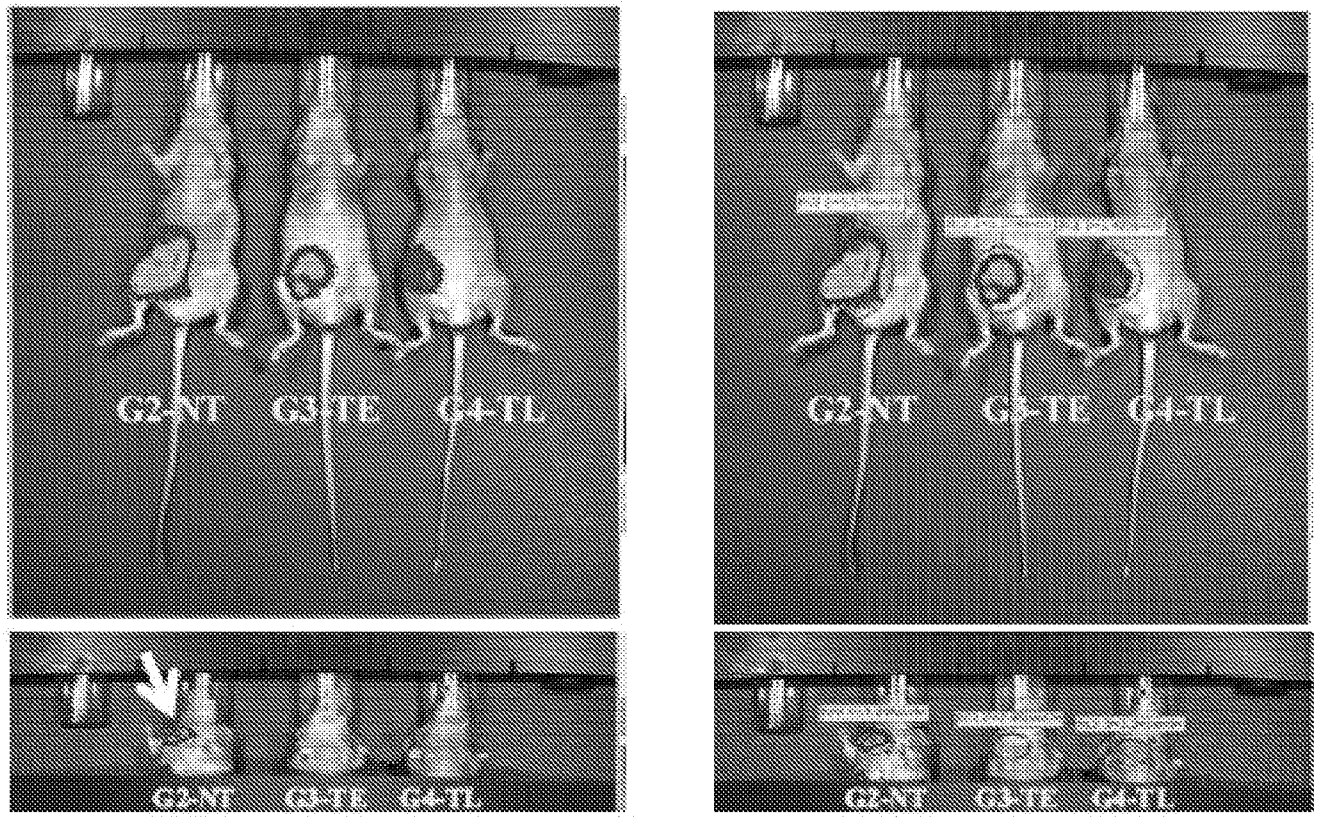



Figure 5B

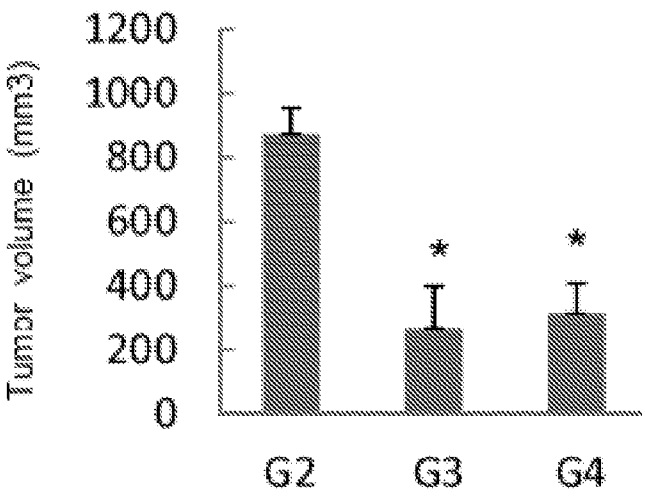



Figure 5C

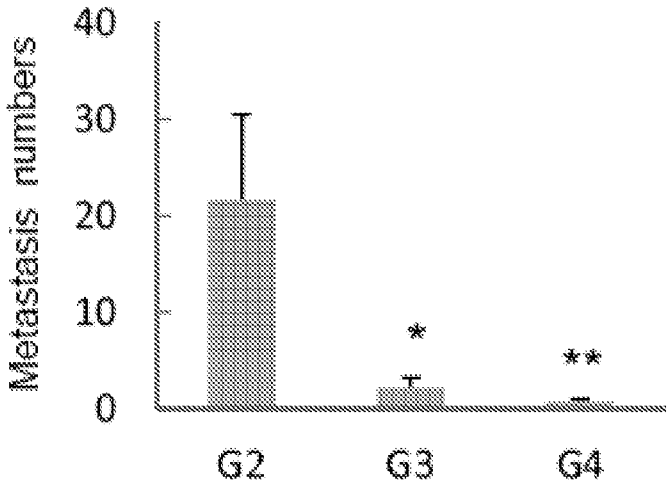


Figure 6

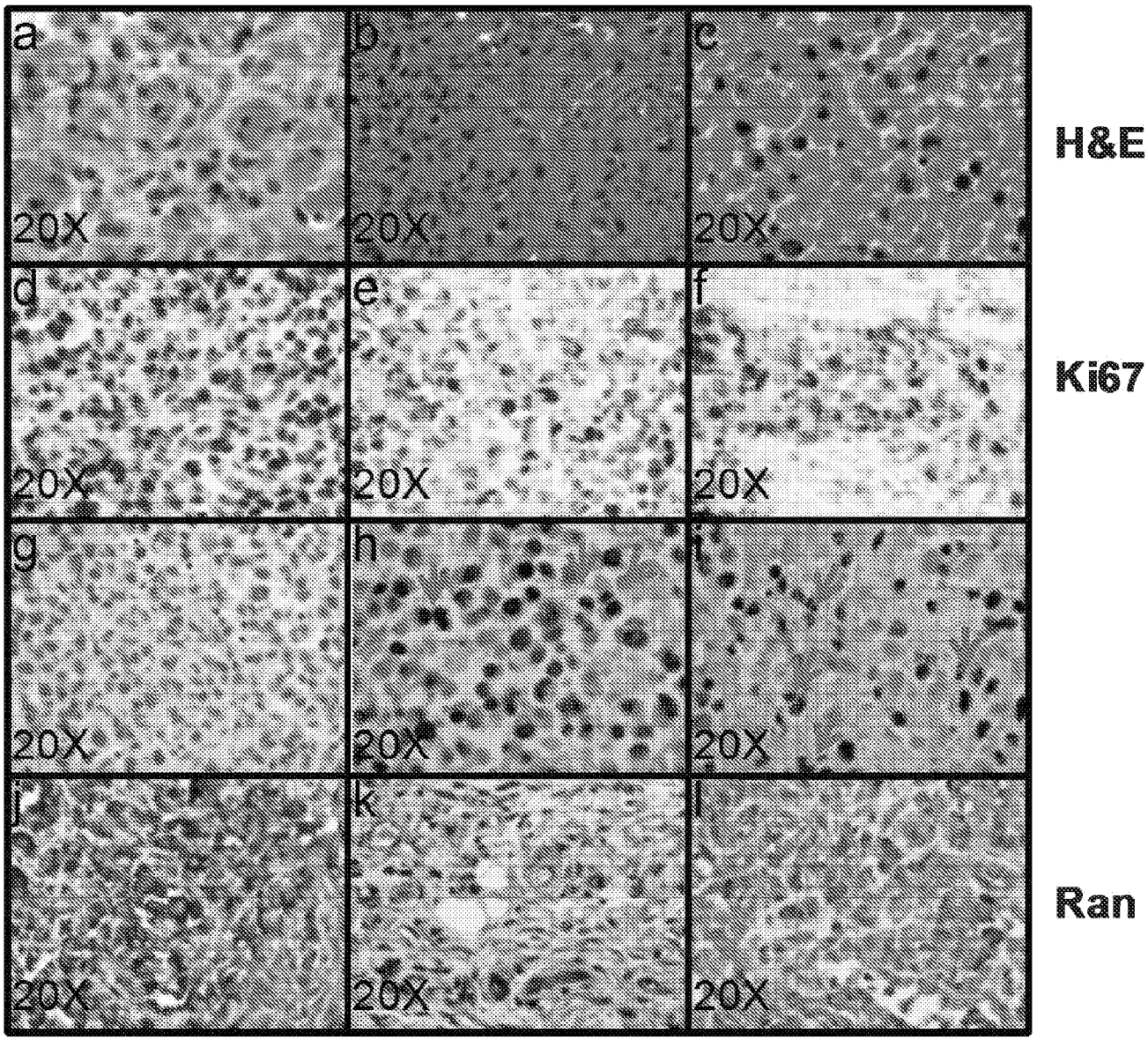

a
20X
b
20X
c
20X
H&E
d
20X
e
20X
f
20X
Ki67
g
20X
h
20X
i
20X
j
20X
k
20X
l
20X
Ran

Figure 7

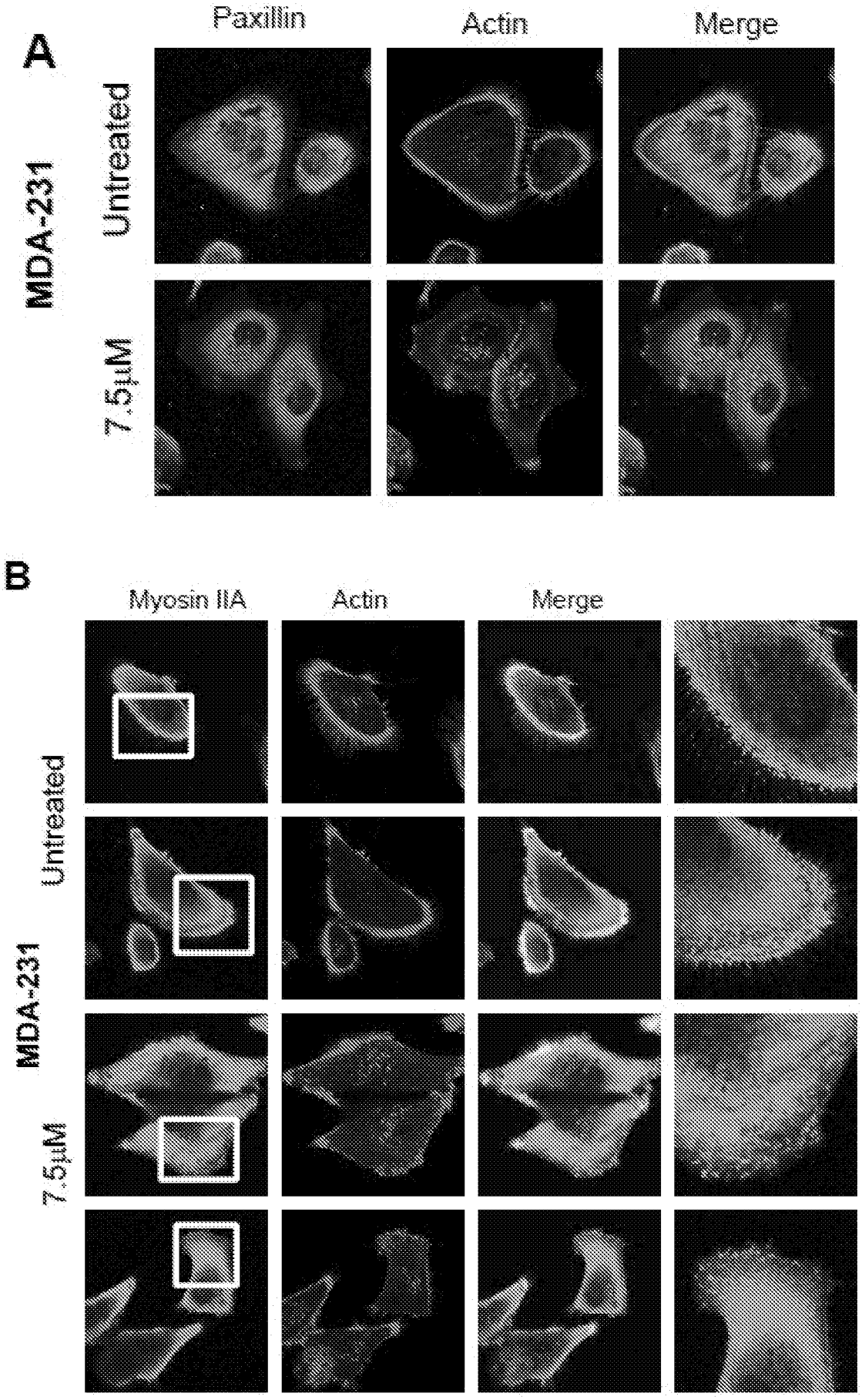

A
Paxillin
Actin
Merge
MDA-231
Untreated
7.5μM
B
Myosin IIA
Actin
Merge
MDA-231
Untreated
7.5μM

Figure 7 (cont.)

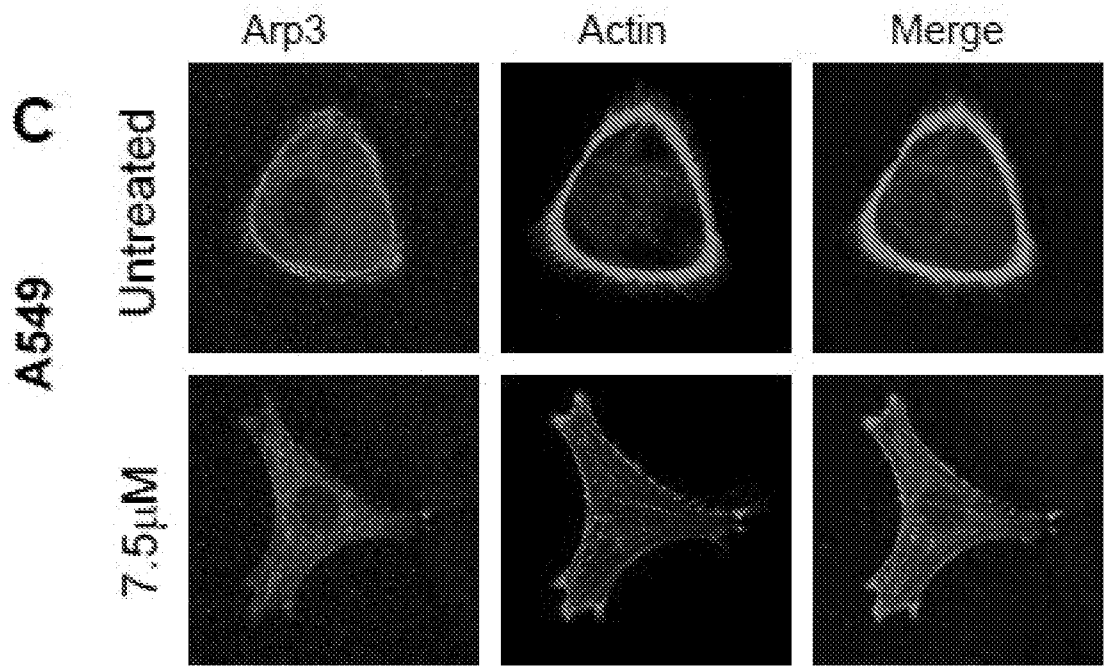


**Figure 8**

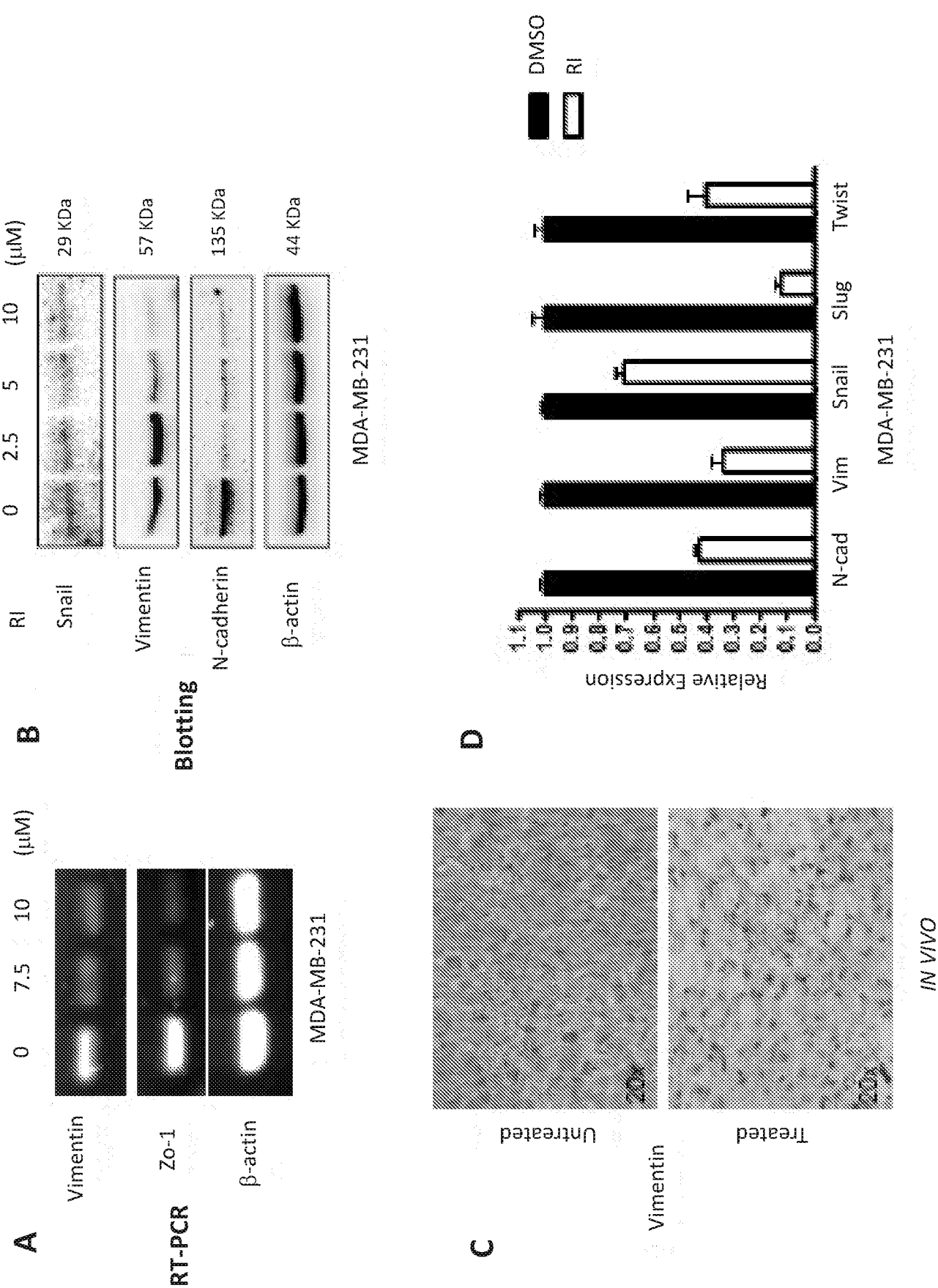

A
RT-PCR
Vimentin
Zo-1
β-actin
0 7.5 10 (μM)
MDA-MB-231
B
Blotting
RI
0 2.5 5 10 (μM)
Snail
Vimentin
N-cadherin
β-actin
29 KDa
57 KDa
135 KDa
44 KDa
MDA-MB-231
C
Vimentin
Untreated
Treated
20x
20x
IN VIVO
D
DMSO
RI
Relative Expression
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
N-cad
Vim
Snail
Slug
Twist
MDA-MB-231

Figure 9

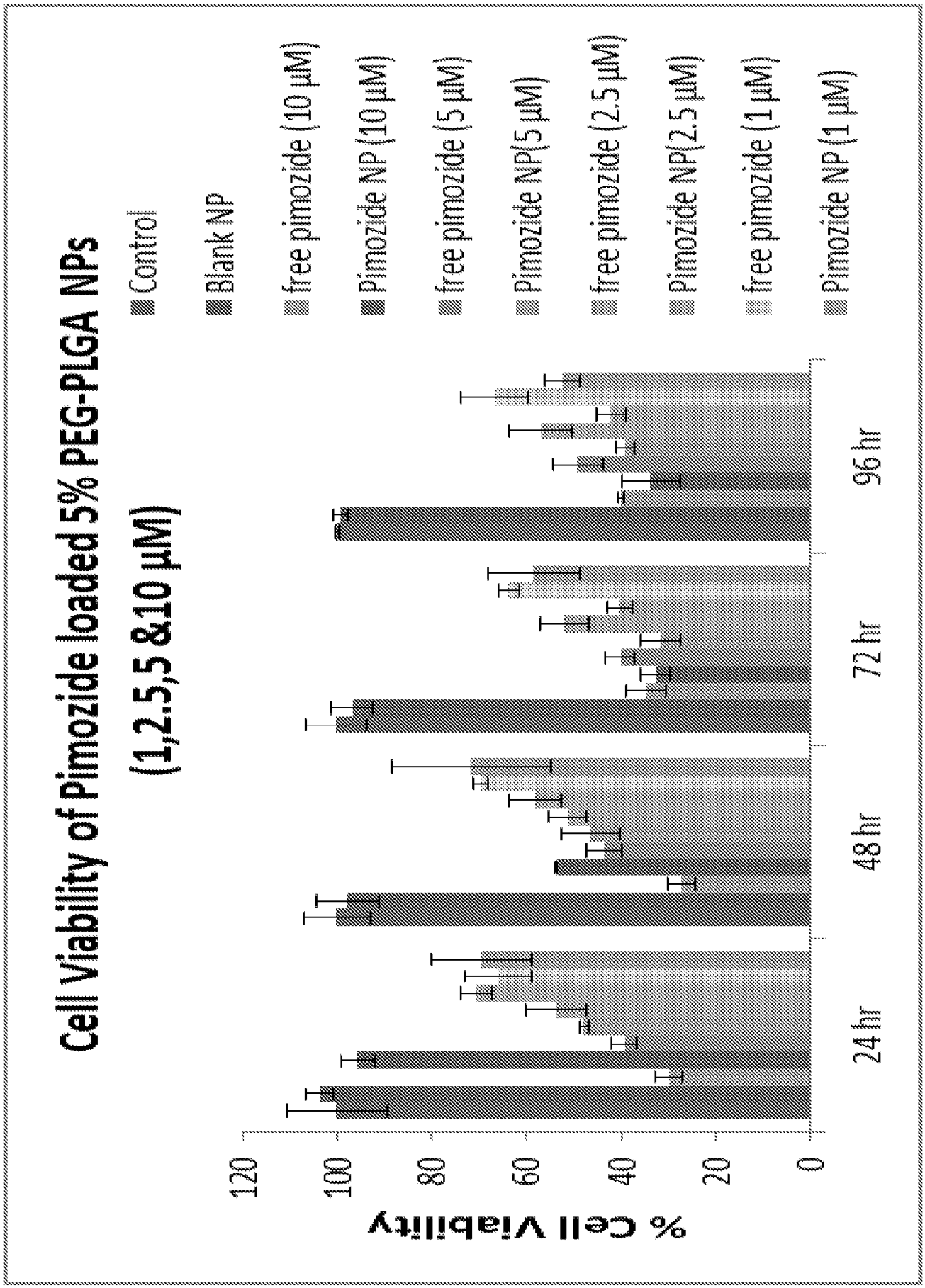
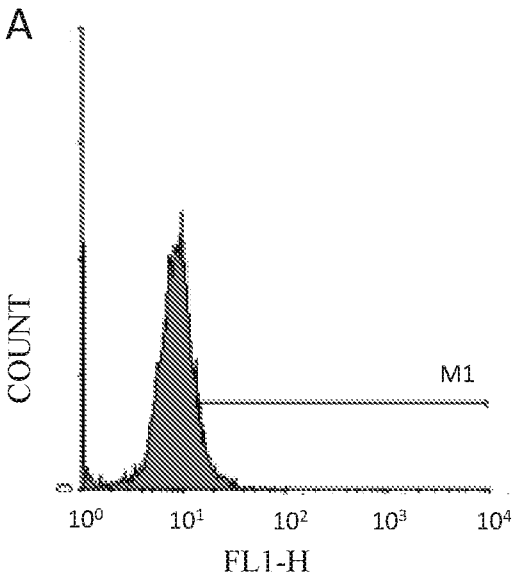
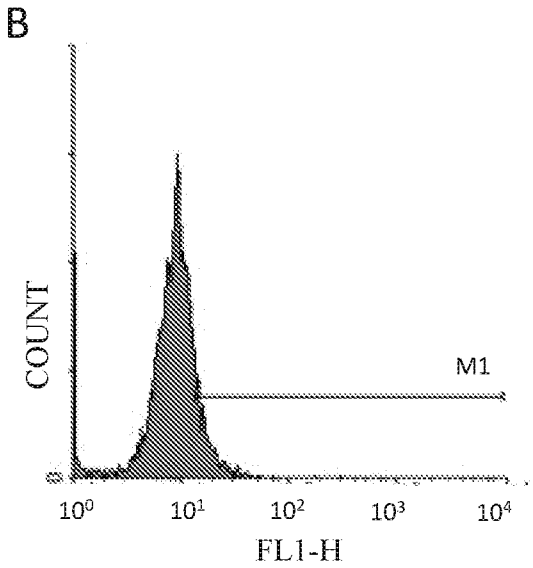

Cell Viability of Pimozide loaded 5% PEG-PLGA NPs
(1,2.5,5 &10 µM)
Control
Blank NP
free pimozide (10 µM)
Pimozide NP (10 µM)
free pimozide (5 µM)
Pimozide NP(5 µM)
free pimozide (2.5 µM)
Pimozide NP(2.5 µM)
free pimozide (1 µM)
Pimozide NP (1 µM)
% Cell Viability
120
100
80
60
40
20
0
24 hr
48 hr
72 hr
96 hr

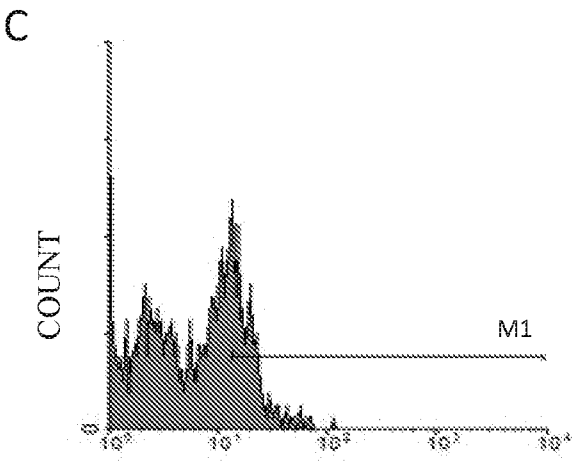
**Figure 10**



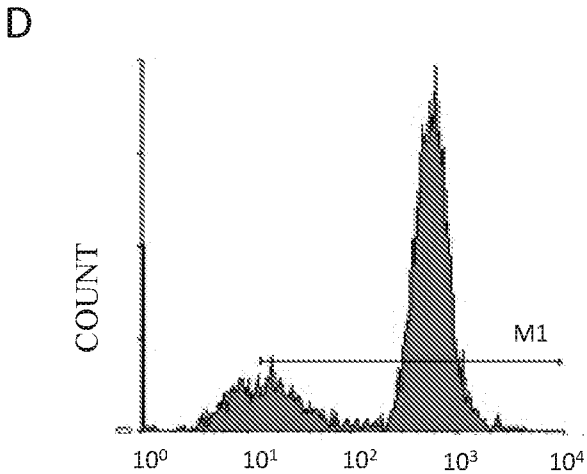

**CONTROL: 4.25%**




**CONTROL-FITC-DYE: 9.42%**




**FITC-DYE-Pimozide-PCL NPs**
**12.16%**




**FITC-DYE-Pimozide-5% PEG-PLGA NPs**
**83.04%**

Figure 11

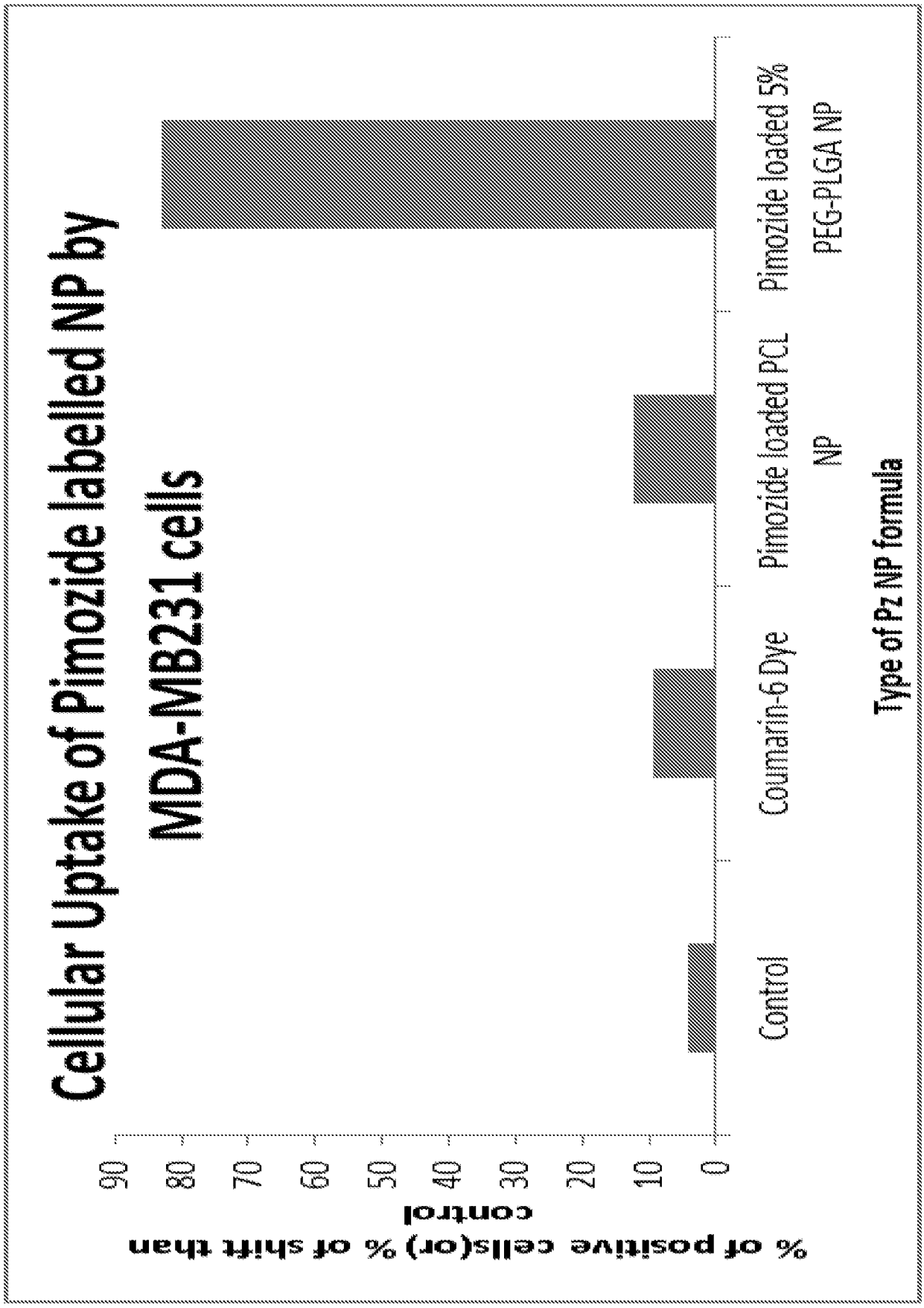
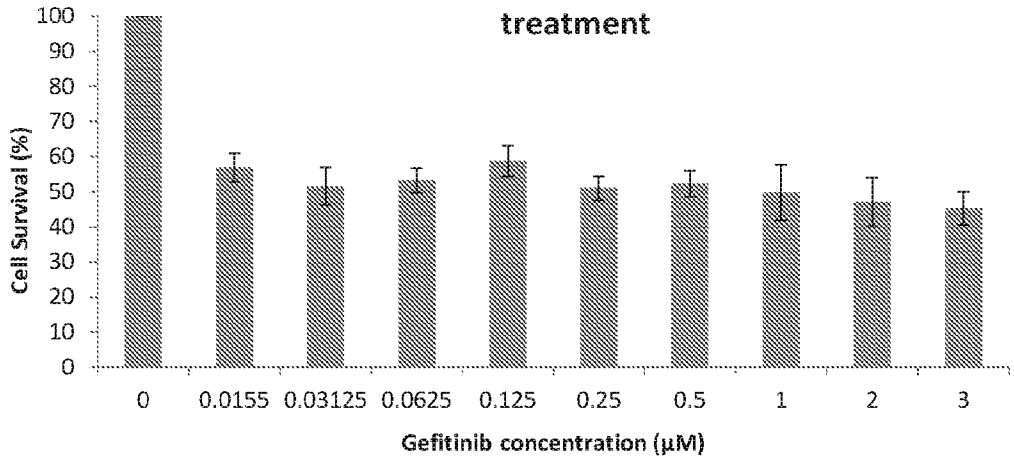

Cellular Uptake of Pimozide labelled NP by MDA-MB231 cells
% of positive cells(or) % of shift than control
0
10
20
30
40
50
60
70
80
90
Control
Coumarin-6 Dye
Pimozide loaded PCL NP
Pimozide loaded 5% PEG-PLGA NP
Type of Pz NP formula

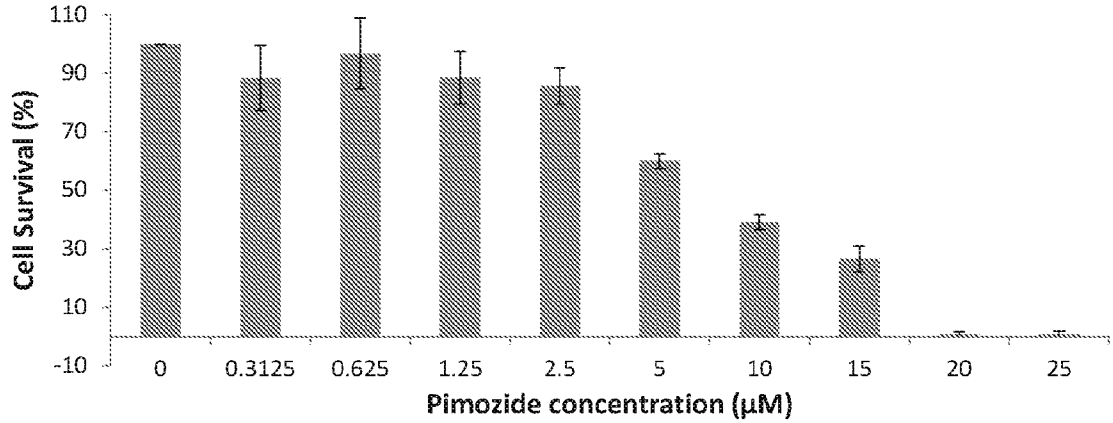
Figure 12



A




B




C

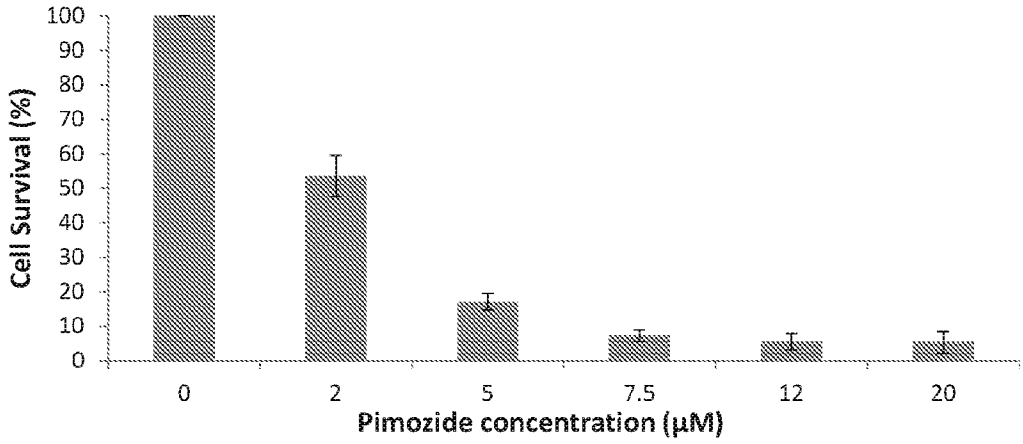
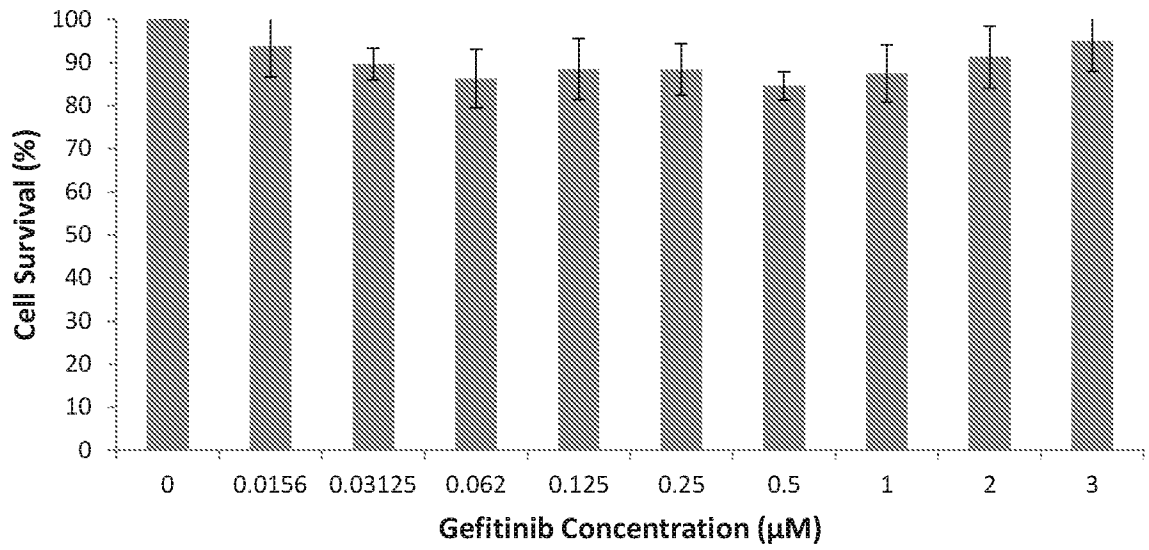
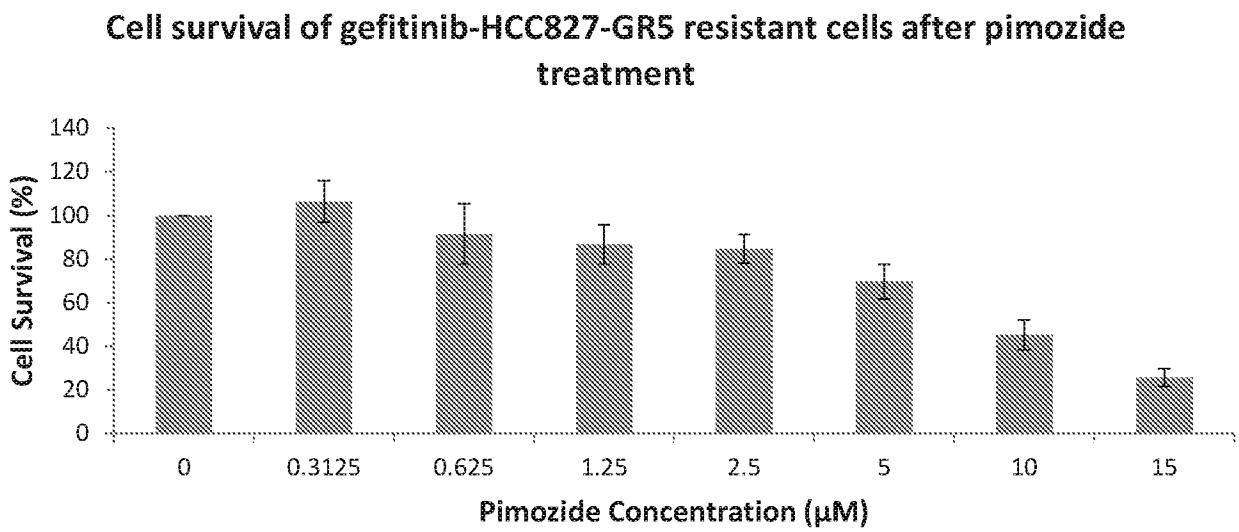


Figure 13

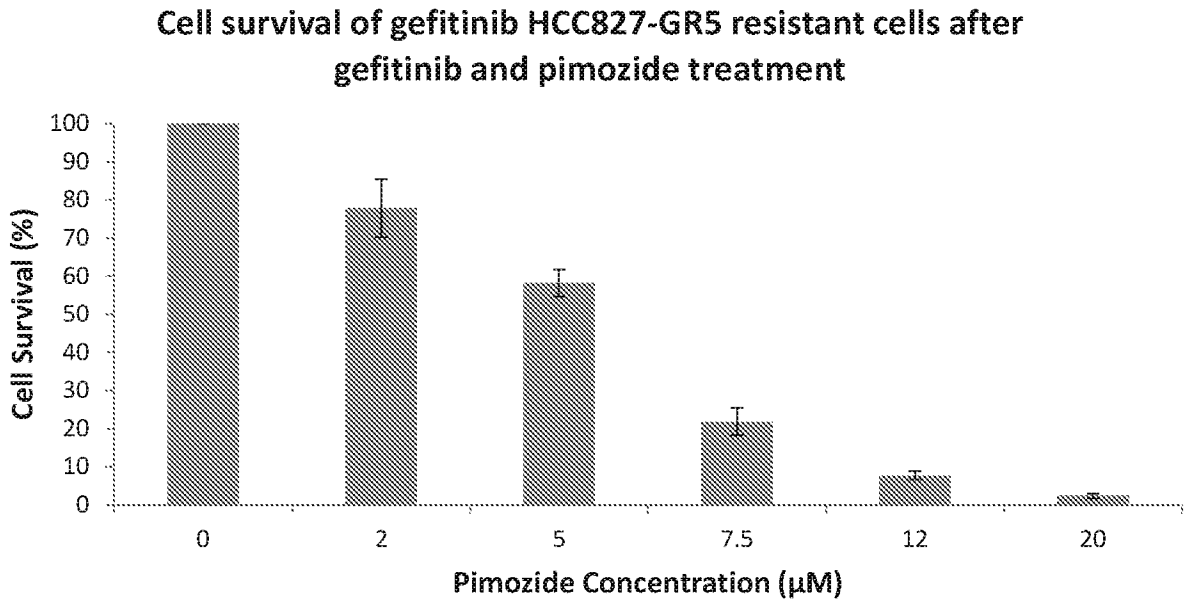
A




B




C



# INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/053009

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/51 A61K31/454 A61K31/5377 A61K45/06 A61P35/00
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br><br><br>Y | WO 2015/158890 A2 (DEUTSCHES KREBSFORSCH [DE]; UNIV MCGILL [CA])<br>22 October 2015 (2015-10-22)<br>the whole document, in particular page 1, paragraph 1; page 9, paragraph 2; page 12, paragraphs 2-3; examples<br>-----<br>-/-- | 1-6,<br>9-12,<br>16-25<br>7-15,26,<br>27 |

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

[X] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 December 2017 | 22/12/2017 |

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

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | DENG LIN ET AL: "Ran GTPase protein promotes human pancreatic cancer proliferation by deregulating the expression of Survivin and cell cycle proteins", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 440, no. 2, 25 September 2013 (2013-09-25), pages 322-329, XP028756289, ISSN: 0006-291X, DOI: 10.1016/J.BBRC.2013.09.079 the whole document | 1-27 |
| | ----- | |
| X | WO 2015/115310 A1 (UNIV KEIO [JP]; SAYA HIDEYUKI [JP]; NAGANO OSAMU [JP]; OKAZAKI SHOGO []) 6 August 2015 (2015-08-06) | 1-6, 9-12, 23-27 |
| Y | the whole document, in particular the examples and the claims | 7,8, 13-15 |
| A | & EP 3 120 849 A1 (UNIV KEIO [JP]) 25 January 2017 (2017-01-25) | 16-22 |
| | ----- | |
| X | WO 2013/160773 A2 (NANOGEN PHARMACEUTICALS [IN]) 31 October 2013 (2013-10-31) | 1,3-13, 23-25 |
| Y | the whole document, in particular page 14, lines 31-34 and page 19, line 26 | 14,15, 26,27 |
| A | | 16-22 |
| | ----- | |
| X | E. A. NELSON ET AL: "The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors", BLOOD, vol. 117, no. 12, 24 March 2011 (2011-03-24), pages 3421-3429, XP055105692, ISSN: 0006-4971, DOI: 10.1182/blood-2009-11-255232 | 1,3-6, 9-12, 23-27 |
| Y | page 504, column 1, paragraph 2 | 13-15 |
| A | page 505, column 1, paragraph 1 - page 506, column 2, paragraph 2 page 508, column 1, paragraph 1 - page 510, column 1, paragraph 2 | 2,7,8, 16-22 |
| | ----- | |
| X | WO 03/009881 A2 (IMARX THERAPEUTICS INC [US]) 6 February 2003 (2003-02-06) | 1,3-13, 25 |
| Y | the whole document, in particular page 12, | 14,15 |
| A | lines 1-7; page 14, lines 14-18; page 16, line 29 - page 17, line 5; page 61, line 4 | 16-24, 26,27 |
| | ----- | |
| | -/-- | |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | BIN YANG ET AL: "Small Angle Neutron Scattering Studies on the Internal Structure of Poly(lactide- co -glycolide)-block -poly(ethylene glycol) Nanoparticles as Drug Delivery Vehicles", BIOMACROMOLECULES, vol. 16, no. 2, 14 January 2015 (2015-01-14), pages 457-464, XP055434746, ISSN: 1525-7797, DOI: 10.1021/bm501519u the whole document, in particular page 457, column 1 | 7,8, 13-15 |
| Y | HIU-FUNG YUEN ET AL: "Ran GTPase promotes cancer progression via Met receptormediated downstream signaling", ONCOTARGET, 3 October 2016 (2016-10-03), XP055382563, United States ISSN: 1949-2553, DOI: 10.18632/oncotarget.12420 the whole document, in particular page 75857, column 1, paragraph 2 - page 75860, column 2, paragraph 1 | 26,27 |
| Y | XIAOHUA ZHOU ET AL: "Gefitinib Inhibits the Proliferation of Pancreatic Cancer Cells via Cell Cycle Arrest", THE ANATOMICAL RECORD : AR ; ADVANCES IN INTEGRATIVE ANATOMY AND EVOLUTIONARY BIOLOGY, vol. 292, no. 8, August 2009 (2009-08), pages 1122-1127, XP055434917, Hoboken, NJ : Wiley-Liss ISSN: 1932-8486, DOI: 10.1002/ar.20938 the whole document, in particular figure 1 | 9-12,26, 27 |

3

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2017/053009

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2015158890 A2 | 22-10-2015 | CA 2945635 A1 | 22-10-2015 |
| | | EP 2932969 A1 | 21-10-2015 |
| | | EP 3131548 A2 | 22-02-2017 |
| | | US 2017035746 A1 | 09-02-2017 |
| | | WO 2015158890 A2 | 22-10-2015 |
| WO 2015115310 A1 | 06-08-2015 | EP 3120849 A1 | 25-01-2017 |
| | | JP 6101827 B2 | 22-03-2017 |
| | | JP 2017031059 A | 09-02-2017 |
| | | JP WO2015115310 A1 | 23-03-2017 |
| | | US 2017105984 A1 | 20-04-2017 |
| | | WO 2015115310 A1 | 06-08-2015 |
| WO 2013160773 A2 | 31-10-2013 | EP 2841486 A2 | 04-03-2015 |
| | | JP 2015525245 A | 03-09-2015 |
| | | US 2015353676 A1 | 10-12-2015 |
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| WO 03009881 A2 | 06-02-2003 | AU 2002330886 A1 | 17-02-2003 |
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| | | US 2002041898 A1 | 11-04-2002 |
| | | WO 03009881 A2 | 06-02-2003 |