The FGFR Inhibitor NVP-BGJ398 Induces NSCLC Cell Death by Activating Caspase-dependent Pathways as well as Caspase-independent Apoptosis

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Abstract. *Background: Fibroblast growth factor receptors are expressed in diverse cell types. They play a critical role in tumor development. Their activation promotes cell-cycle progression, angiogenesis, and cell survival by induction/suppression of the expression of proteins involved. Materials and Methods: Non-small cell lung cancer (NSCLC) cells (line H1581) were treated with NVP-BGJ398 to evaluate effects on growth by western blot, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay and cell-cycle analysis. Results: NVP-BGJ398 induced cell death in H1581 cells by activating caspase-dependent mitochondrial and non-mitochondrial pathways. Caspase-independent apoptosis was also activated. Cells were found to be arrested in the G_0/G_1 phase. Furthermore, the expression of the tumor-suppressor gene programmed cell death 4 (PDCD4) was up-regulated with suppression of angiopoietin 2 (ANG2). This represents an additional mechanism by which NVP-BGJ389 inhibits tumor growth. Conclusion: Various pathways induce apoptosis in NSCLC cells by employing NVP-BGJ398. These data reflect the potential of cancer treatment utilizing small FGFR inhibitors.*

Fibroblast growth factor receptors (FGFR) 1-4 are expressed in diverse cell types and play a critical role in tumor development. After ligand binding, a series of downstream signalling pathways is activated, often in a highly cell type-specific manner. The FGFRs have two important substrates, fibroblast growth factor receptor substrate 2 (FRS2) and phospholipase-Cγ (PLCγ) (7). FRS2 can activate several pathways including two most important ones, in the context of cancer the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3-kinase/protein kinase B (PI3K)/AKT) pathways. Phospholipase C binds directly to FGFR Y766 and is then phosphorylated by FGFR. Both pathways promote cell-cycle progression, angiogenesis and cell survival by induction or suppression of the expression of various proteins involved in these processes.

FGFR- activating mutations and overexpression are known to play an important role in the development of various types of cancers such as bladder , liver cancer, multiple myeloma and renal cell carcinoma (15). A very important aberration of FGFR1 is genetic amplification, that has been found in several cancer types, such as squamous cell lung cancer and breast cancer. Therefore, inhibition of FGFR may be a promising tool in cancer treatment. Weiss *et al*. demonstrated that a non-small cell lung cancer (NSCLC) cell line (H1581) can be inhibited in growth and forced into apoptosis by the small molecule inhibitor PD173074 (17). Considering these facts, the interest in FGFR inhibitors as anticancer agents is continuously increasing.

FGFR inhibitors can be divided into two groups, according to their mechanisms of action. There are macromolecular antibodies or peptide inhibitors, which bind to the extracellular domain of the receptor and thus block the FGF–FGFR1 assembly and FGFR dimerization. The second group contains small molecules, which bind to various cytoplasmatic regions of the receptor and inhibit its autophosphorylation and catalytic activity (10). More and more specific inhibitors targeting FGFRs are becoming commercially available and are being tested in various

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studies. Some of the inhibitors have entered clinical trials, *e.g.* the small molecule NVP-BGJ398, which we used in our study (10).

Many studies have shown a decrease in growth and an increase of apoptosis only with limited procedures such as non-specific annexin V staining or detection of cleaved caspase-3 by western blot (14, 17). We aimed to evaluate in more detail the effects of the FGFR1 inhibitor NVP-BGJ398 on the NSCLC cell line H1581 which is known to overexpress FGFR1 protein and be sensitive to the inhibition of FGFR1 by small-molecule inhibitors. For cancer treatment, it is essential to understand the mechanisms underlying the apoptosis induced by FGFR1 inhibitors. In addition to the evaluation of this pathway, we intended to learn more on the role of this inhibitor in cell-cycle arrest.

Materials and Methods

Cell culture. H1581 (human NSCLC) cells were cultured in RPMI-1640 complete media (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum. The cell line was obtained from American Type Culture Collection.

The specific FGFR inhibitor 3-(2,6-dichloro-3,5-dimethoxy-phenyl)-1-6-[4-(4-ethyl-piperazin-1-yl)-phenylamino]-pyrimidin-4-yl-1-methyl-urea (NVP-BGJ398) was purchased from Novartis (Nürnberg, Germany), dissolved as a stock solution in dimethyl sulfoxide (DMSO: Carl Roth GmbH, Karlsruhe, Germany) and stored at −20˚C in aliquots. Prior to use 1 μl of the stock solution was diluted with phosphate-buffered saline (PBS) to yield a 10 μM solution used for the treatments.

Inhibition assay. H1581 cells were treated with NVP-BGJ398 inhibitor to evaluate the growth effect on this cell line. Firsty, cells were plated to 50% confluency in 6 cm dishes with 5 ml of media and left overnight to adhere. Treatment was then performed for 48 h with and without inhibitor at different concentrations (0.05 μM, 0.1 μM and 0.2 μM) established by an inhibition curve.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Plated cells (H1581) were grown in a 12-well plate in 1 ml of media for 1, 2 and 3 days or grown for 1, 2 and 3 days and incubated in the absence or presence of NVP-BGJ398 (0.05 μM, 0.1 μM and 0.2 μM) with or without 100 ng/ml recombinant human FGF acidic aa2-155 (R&D Systems, Minneapolis, MN, USA). Cell proliferation was determined by MTT assay. Briefly, growth medium was replaced by medium without serum, 100 μl of MTT reagent (5 mg/ml PBS, Carl Roth GmbH) were added to the cells and the incubation was continued for 2 h. After removal of the medium, cells were lysed with 200 μl lysis buffer (80 ml isopropanol, 10 ml triton-X100, 10 ml 1M HCl) for 10 minutes. The absorbance of 50 μl of the samples was then determined at 570 nm in a 96-well plate using a multiwell plate reader.

Cell fractionation. Treated and untreated cells were washed twice in PBS (Life Technologies) and suspended in hypotonic buffer A (20 mM HEPES-KOH, pH=7.5; 10 mM KCl; 1 mM EGTA; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonylfluoride), incubated for 15 minutes on ice and passed 10 times through a 21-gauge needle. Cell homogenate was centrifuged for 10 minutes at 4˚C at 600 × *g*. The pellet was resuspended in buffer A (see above) to yield nucleic proteins and the supernatant was centrifuged for 1 hour at 4˚C at 100,000 × *g* for cytosolic proteins. Equal amounts of protein (20 μg) were loaded onto the gels.

Western blot and antibodies. Total proteins were extracted from cells by sonication and protein concentration was determined using a protein assay according to the manufacturer's instructions (Bio-Rad). Equal amounts of protein were loaded onto sodiumdodecylsulfate polyacrylamide gels. After the gel run, the proteins were electrotransferred on to a nitrocellulose membrane (pore size 0.45 or 0.2 μm). To verify equal loading of the proteins Ponceau S staining and detection of β-actin was used. Immunodetection was carried out by probing the membrane with different antibodies in 5% non-fat dried milk powder (Carl Roth GmbH) (or 5% bovine serum albumin, Sigma-Aldrich, St. Louis, MO, USA), 50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 overnight. The next day appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were left on the membrane for 1 hour. Western-blot signals were detected by using an enhanced chemiluminescence (ECL), ECL+ or ECL Advance kits (GE Health Care Life Sciences, Munich, Germany). The antibodies were purchased from Alpha Diagnostics (Santa Monica, CA, USA): Angiopoetin-2 (ANG2), Cell Signalling (Danvers, MA, USA): phophso-p44/42 MAPK (ERK1/2;Thr202/Tyr204), cytochrome c, apoptosis-inducing factor (AIF), caspase-8 1C12, cleaved caspase-3, caspase-3, caspase-2, cyclin-dependent kinase 4 (CDK4), pAKT (Ser472), BH3 interacting domain death agonist (BID), Poly (ADP-ribose) polymerase (PARP), cell division cycle protein 2 (CDC2), retinoblastoma protein (pRB), cyclin D3, cyclin A2, cyclin-dependent kinase 6 (CDK6), Santa Cruz (Dallas, TX, USA): B-cell lymphoma 2 (BCL2)-HRP, caspase-9, BCL-2-associated X protein (BAX)-HRP, and (NF-κB) p65.

Cell cycle distribution. H1581 cells were seeded at a density of 80.000 cells per well in a 12-well plate. Cells were treated with the FGFR1 inhibitor NVP-BGJ398 at a concentration of 50, 100, or 200 nM for 48 h or 72 h. Cells were harvested at the different time points using trypsin, washed with PBS and stained with Nicoletti buffer for 30 min. Cell-cycle distribution was measured using a BD Accuri C6 flow cytometer (Becton-Dickinson, Heidelberg, Germany). The events for the subG1, G_0/G_1, S, and G_2M phase were normalized to the total number of events. Measurements for each concentration and time point were performed in triplicates.

Results and Discussion

Since FGFR-activating mutations and overexpression are known to play an important role in the development of various cancer types (15), inhibition of FGFR may be a promising tool for cancer treatment. Recently, it was demonstrated that a NSCLC cell line (H1581) can be inhibited in growth and forced into apoptosis by the small molecule inhibitor PD173074 (17). To test whether the pro-apoptotic effect of small- molecule inhibitors are side-effects or due to inhibition of FGF–FGFR1 pathway, we used the

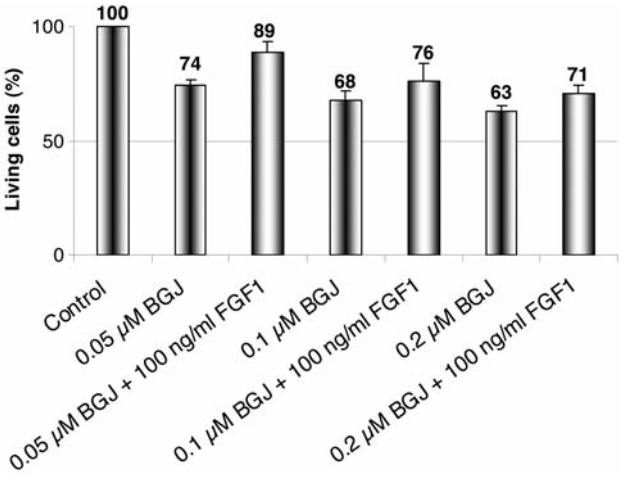

Figure 1. *NVP-BGJ398 (BGJ) reduces cell viability in H1581 cells which is attenuated by fibroblast growth factor-1 (FGF1). H1581 cells were incubated for 24 h in the presence and absence of NVP-BGJ398 with or without FGF1. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay as described in the Materials and Methods. Data are representative of at least three independent experiments.*

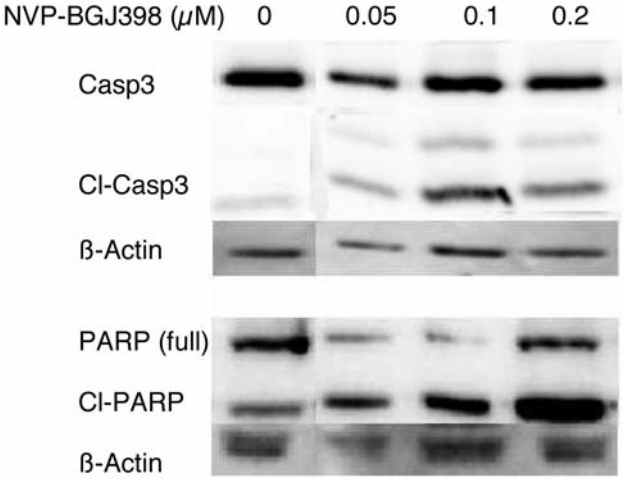

Figure 2. *NVP-BGJ398 induces cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) in H1581 cells. Cells were treated in the absence and presence of NVP-BGJ398 for 48 h. Apoptosis was analyzed by western blot with antibodies directed against caspase-3 (Casp3) and PARP. Cl: Cleaved. Data are representative of at least three independent experiments.*

small- molecule FGFR inhibitor NVP-BGJ398 which already entered clinical trials for our experiments. Incubation of H1581 cells with NVP-BGJ398 for 24 h reduced cell viability which was clearly attenuated in the presence of FGF1 (Figure 1), suggesting specific inhibition of FGF-FGFR1 pathway by the inhibitor.

There are different routes leading to programmed cell death. The best investigated are the extrinsic and intrinsic apoptotic pathways which merge at the caspase-3 level (13). To examine whether NVP-BGJ398 induced apoptosis is caspase-dependent, H1581 cells were incubated with a range of concentrations of the inhibitor for up to 48 h. Analyses of cell protein extracts revealed caspase-3 cleavage in the presence of the inhibitor (Figure 2). Furthermore, PARP a downstream target of caspase-3 was cleaved in cells treated with NVP-BGJ398 (Figure 2). To further elucidate the apoptotic pathway activated by NVP-BGJ398, H1581 cells were incubated with a range of concentrations of the inhibitor for up to 48 h. As shown in Figure 3a, inhibition of FGFR1 by NVP-BGJ398 induced cleavage of the initiator caspase-2 but had no effect on caspase9 or caspase7 (Figure 3a).

The mechanisms by which caspase-2 is activated are unclear. Dimerization might be the key event driving initial caspase-2 activation, followed by autolytic cleavage that promotes stable dimerization and enhances autolytic activity (2). However, it is not known whether NVP-BGJ398 has an effect on dimerization of caspase-2. There is also evidence that caspase 2 is activated by endoplasmic reticulum stress (16). In this context, it is of special interest that FGFR1 has an impact on inositol trisphosphate production and intracellular Ca^{2+} levels *via* regulation of PLC-γ activity (3). Inhibition of FGFR1 by NVP-BGJ398 may induce endoplasmic reticulum stress by altering intracellular calcium levels.

Caspase-2 has been reported to cleave mitochondrial BID (16). Indeed, we found that incubation of H1518 cells with NVP-BGJ398 reduced the level of full-length BID (Figure 3a). However, there was no cytochrome *c* release detectable and BAX and BCL2 levels were unchanged (Figure 3b).

Recently, sequential activation of caspase2 and caspase8 was reported in saikosaponin- as well as in ceramide- and etoposide-induced apoptosis (6, 11). In accordance with these data, we show that incubation of H1581 cells with NVP-BGJ398 resulted in a cleavage of caspase-8 indicating that NVP-BGJ398 activates both mitochondrial and non-mitochondrial apoptotic pathways (Figure 4).

AIF is a key trigger of caspase-independent apoptosis (12). Since NVP-BGJ398 induces caspase-2 activation and caspase-2 can directly induce AIF release from mitochondria (5), we investigated whether AIF is released from mitochondria in response to the FGFR1 inhibitor. After incubation of H1581 cells for 48 h with and without NVP-BGJ398, protein extracts were prepared and analyzed for the presence of AIF (Figure 5a). AIF was increased in the cytosol of cells treated with NVP-BGJ398, indicating that NVP-BGJ398 induces both caspase-dependent and independent apoptosis.

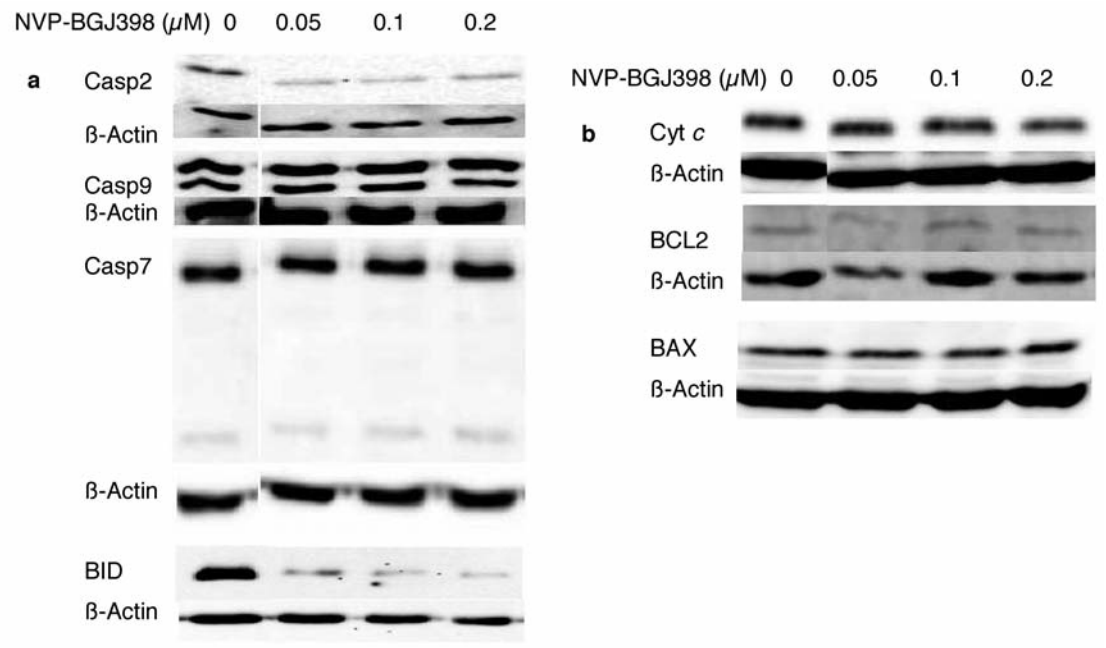

Figure 3. *H1581 cells were treated in the absence and presence of NVP-BGJ398 for 48 h. Protein expression was analysed by western blots with antibodies directed against caspase (Casp)-2, -7and -9, and BH3 interacting domain death agonist (BID) (a) and B-cell lymphoma 2 (BCL2), Bcl-2-associated X protein (BAX) and cytochrome c (b). NVP-BGJ398 induced cleavage of caspase-2 but not of caspase-9 and -7, and reduced BID expression in H1581 cells (a). However, NVP-BGJ398 did not change expression of BCL2, BAX and cytochrome c (b). Data are representative of at least three independent experiments.*

Recently, it has been shown that FGF1 targets the NF-κB pathway in haematopoietic stem and progenitor cells (18). Furthermore, AKT was reported to regulate NF-κB activity by controlling its sub-cellular localization (4). However, we found that incubation of H1581 cells with NVP-BGJ398 had no effect on pAKT (Figure 5a), and did not alter the subcellular localization of NF-κB (Figure 5b). This suggests that FGF1 does not target the NF-κB pathway in H16581 cells.

In order to avoid unwanted unspecific side-effects, we decided to cautiously employ lower inhibitor concentrations compared to previous groups (17). Nonetheless, we still observed obvious changes in pERK1/2, the downstream target of FGFR1, using the inhibitor NVP-BGJ398 (Figure 5a).

To further investigate how the cell cycle is altered, we incubated H1581 cells for 48 h with and without NVP-BGJ398. Analyses of cytosol preparations showed decreased protein levels for CDK4 and cyclin D1 (Figure 5c). Furthermore, cell-cycle distribution was investigated by fluorescence-activated cell sorting analysis after incubation of H1581 cells for up to 48 h with and without NVP-BGJ398. Treatment of cells with NVP-BGJ398 resulted in an arrest of the cell cycle at the G_0/G_1 phase. The proportion of cells in the G_0/G_1 phase was 58% without treatment and went up significantly at 50 nM of NVP-BGJ398 and

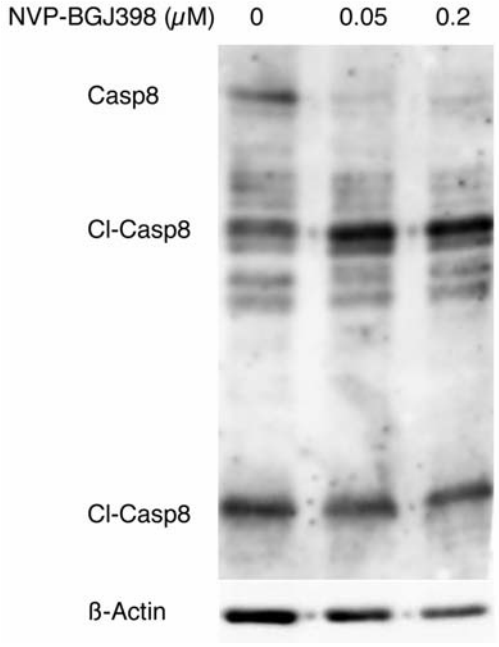

Figure 4. *NVP-BGJ398 induces cleavage of caspase-8 (Casp8) in H1581 cells. Cells were treated in the absence and presence of NVP-BGJ398 for 48 h. Protein expression was analysed by western blot with antibodies directed against caspase-8 (Casp8). Cl: Cleaved. Data are representative of at least three independent experiments.*

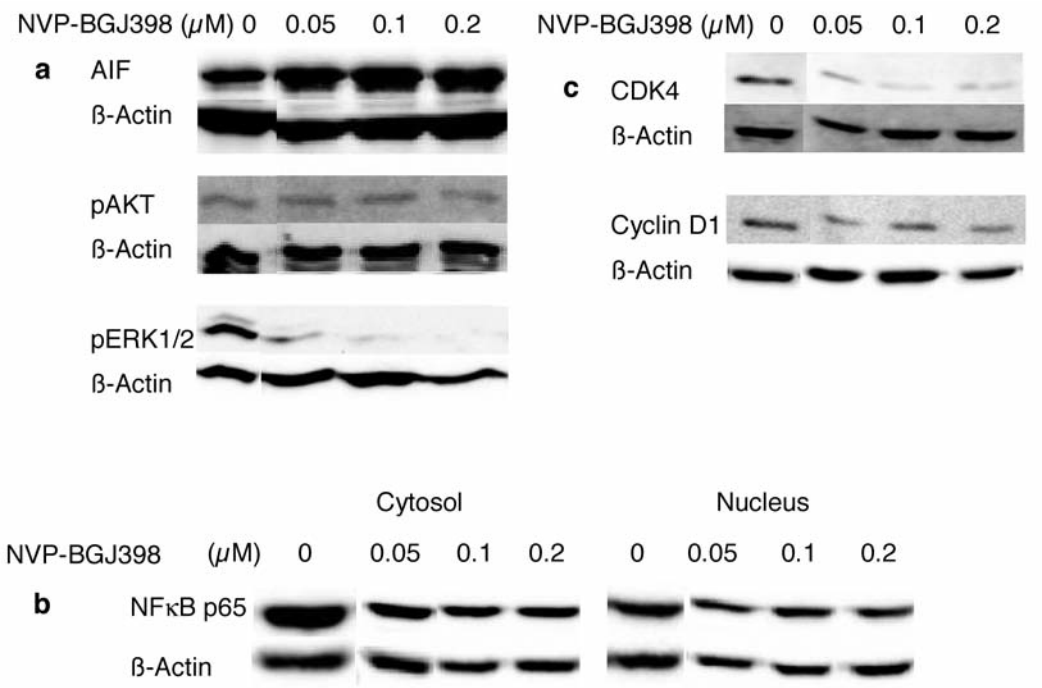

Figure 5. *Cells were treated in the absence and presence of NVP-BGJ398 for 48 h. Protein expression was analysed by western blot with antibodies directed against apoptosis-inducing factor (AIF), protein kinase B (pAKT) extracellular-signal-regulated kinase (pERK1/2) (a), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (b) and cyclin-dependent kinase 4 (CDK4) and cyclin D1 (c). NVP-BGJ398 increased the expression of AIF but had no effect on the expression of pAKT, while reducing that of pERK1/2 (a). The subcellular localization of NF-κB in H1581 cells was not affected by NVP-BGJ398 (b). Expression of CDK4 and cyclin D1 was reduced by NVP-BGJ398. Data are representative of at least three independent experiments.*

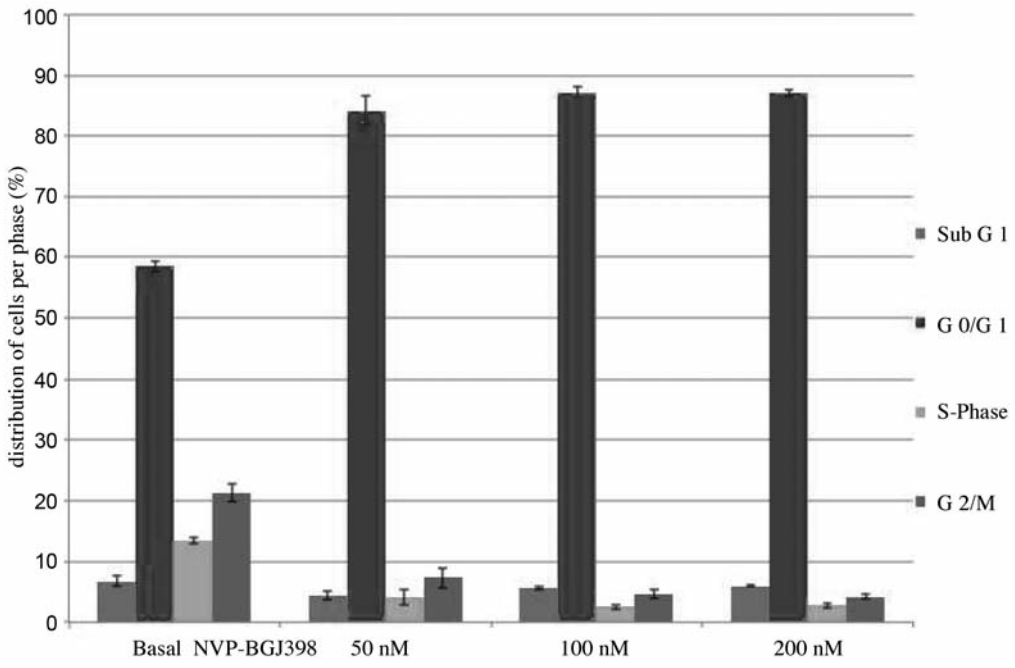

Figure 6. *NVP-BGJ398 induces arrest of H1581 cells in G_0/G_1 phase of cell cycle. H1581 cells were incubated for 48 h in the absence and presence of a range of NVP-BGJ398 concentrations. Cell cycle distribution was analysed as described in the Materials and Methods. Data are representative of at least three independent experiments.*

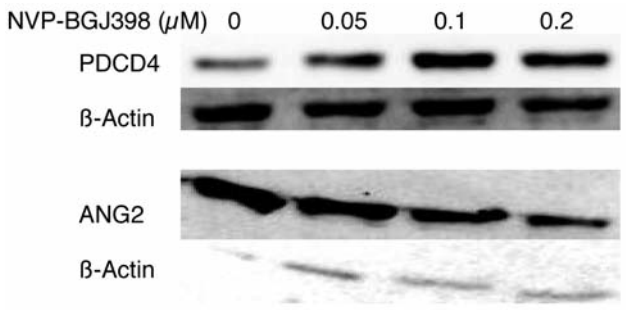

Figure 7. *NVP-BGJ398 increases expression of programmed cell death 4 (PDCD4) and reduces expression of angiopoietin 2 (ANG2). H1581 cells were treated in the absence and presence of NVP-BGJ398 for 48 h. Protein expression was analysed by western blots with antibodies directed against PDCD4 and ANG2. Data are representative of at least three independent experiments.*

eventually amounted to 87% when adding 200 nM of the inhibitor (Figure 6).

PDCD4 is a well- established tumor-suppressor gene that is regulated by different pathways (9). Previously, using several *PDCD4* knock-down cell lines we succeeded to identify ANG2 as a gene regulated by *PDCD4*. ANG2 is strongly expressed in many tumors and may promote tumor-associated vascularization. Moreover, elevated levels of ANG2 were detected in the circulation of patients with cancer and possibly the increase in ANG2 concentration correlates with tumor progression (1). Knock-down of *PDCD4* resulted in an explicit increase of *ANG2* mRNA and protein levels accompanied by enhanced release. Reduction of PDCD4 levels in neuroendocrine Bon-1 cells resulted in an enhanced peptide secretion including ANG2 which forced wild-type cells in a neoplastic direction demonstrated by increased proliferation and colony formation while cell adhesion was reduced (8). In the present work, we showed that NVP-BGJ389 up-regulates *PDCD4* expression in H1581 cells with a down-regulation of *ANG2* (Figure 7). This suggests an additional mechanism by which NVP-BGJ389 exerts its antineoplastic activity.

In summary, the small-molecule FGFR1 inhibitor NVP-BGJ398 induces cell death in H1581 NSCLC cells by activating caspase-dependent mitochondrial- and non-mitochondrial pathways, as well as by activating caspase-independent apoptosis and arresting cells in the G_0/G_1 cell cycle phase. Furthermore it up-regulates the expression of the tumor-suppressor gene *PDCD4* with a suppression of ANG2 which may represent an additional mechanism by which NVP-BGJ389 inhibits tumor growth

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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