GSK3 / : A Novel Therapeutic Target for Neuroendocrine Tumors

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Abstract: INTRODUCTION Glycogen synthase kinase 3 / (GSK3 /) is a serine/threonine kinase that plays a critical role in cancer. AIMS In this study, we evaluated the effects of the specific GSK3 / inhibitor AR-A014418 in vitro to gain novel insights into GSK3 / signaling in neuroendocrine tumors (NETs). MATERIALS AND METHODS Human NET cell lines (BON1, QGP1, H727, and GOT1) were treated with different concentrations of AR-A014418 alone and in combination with lovastatin, everolimus, 5-fluorouracil (5-FU), and -irradiation. RESULTS AR-A014418 significantly dose- and time-dependently decreased cell viability in all 4 NET cell lines through inhibition of epithelial growth factor receptor and mTORC1/p70S6K signaling, as well as cyclin D3 downregulation and induction of pChk1. In all cell lines tested, FACS analysis showed an AR-A014418-induced increase in the sub-G1 phase, reflecting cell death. Apoptosis induction was observed in H727, GOT1 and QGP1 cells, but not in BON1 cells. Furthermore, significant antimigratory effects upon GSK3 / inhibition were found and were associated with -catenin downregulation in all cell lines tested. Compensatory upregulation of pAkt and pERK in response to GSK3 / inhibition was prevented by combining AR-A014418 with the ERK and Akt inhibitor lovastatin. Accordingly, the lovastatin/AR-A014418 combination was synergistic in BON1 and QGP1 cells. Moreover, AR-A014418 displayed promising chemosensitizing effects on 5-FU in QGP1 and slight radiosensitizing properties in BON1 and QGP1 cells. CONCLUSION Our data provide new insights into the role of GSK3 / in NETs and suggest that GSK3 / inhibition could be a novel therapeutic option in NETs, especially in combination with lovastatin or 5-FU, depending on tumor entity.

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GSK3 α / β : A Novel Therapeutic Target for Neuroendocrine Tumors?

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Keywords

GSK3 α / β inhibition · Neuroendocrine tumors · Combination treatment

Abstract

Introduction: Glycogen synthase kinase 3 α / β (GSK3 α / β) is a serine/threonine kinase that plays a critical role in cancer. **Aims:** In this study, we evaluated the effects of the specific GSK3 α / β inhibitor AR-A014418 in vitro to gain novel insights into GSK3 α / β signaling in neuroendocrine tumors (NETs). **Materials and Methods:** Human NET cell lines (BON1, QGP1, H727, and GOT1) were treated with different concentrations of AR-A014418 alone and in combination with lovastatin, everolimus, 5-fluorouracil (5-FU), and γ -irradiation. **Results:** AR-A014418 significantly dose- and time-dependently decreased cell viability in all 4 NET cell lines through inhibition of epithelial growth factor receptor and mTORC1/p70S6K signaling, as well as cyclin D3 downregulation and induction of pChk1. In all cell lines tested, FACS analysis showed an AR-A014418-induced increase in the sub-G1 phase, reflect-

ing cell death. Apoptosis induction was observed in H727, GOT1 and QGP1 cells, but not in BON1 cells. Furthermore, significant antimigratory effects upon GSK3 α / β inhibition were found and were associated with β -catenin downregulation in all cell lines tested. Compensatory upregulation of pAkt and pERK in response to GSK3 α / β inhibition was prevented by combining AR-A014418 with the ERK and Akt inhibitor lovastatin. Accordingly, the lovastatin/AR-A014418 combination was synergistic in BON1 and QGP1 cells. Moreover, AR-A014418 displayed promising chemosensitizing effects on 5-FU in QGP1 and slight radiosensitizing properties in BON1 and QGP1 cells. **Conclusion:** Our data provide new insights into the role of GSK3 α / β in NETs and suggest that GSK3 α / β inhibition could be a novel therapeutic option in NETs, especially in combination with lovastatin or 5-FU, depending on tumor entity.

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Introduction

Neuroendocrine tumors (NETs) are highly complex and often inoperable at the time of diagnosis due to an absence of symptoms and late discovery until metastasis formation [1–3]. Molecularly targeted therapies with the multi-tyrosine kinase inhibitor sunitinib or the mTORC1 inhibitor everolimus are approved for the treatment of pancreatic NETs [4–6]. Everolimus has recently also been approved for lung and nonfunctioning gastrointestinal NET treatment [7]. However, novel therapeutic strategies, including molecularly targeted therapeutics, are urgently needed [6, 8]. A particularly interesting new therapeutic target might be glycogen synthase kinase 3 α/β (GSK3 α/β): GSK3 α/β possesses 2 isoforms (α and β) and, unlike most other kinases, GSK3 α/β is active in its dephosphorylated state and serves as signaling mediator with an impact on diverse cellular pathways [9]. Various studies have demonstrated an association between GSK3 α/β deregulation and tumorigenesis [10, 11]. However, the ambiguous role of GSK3 α/β to act as tumor suppressor in some cases, while presenting oncogenic characteristics in others, has limited the use of GSK3 α/β inhibitors in targeted therapy of cancer so far [10]. In various tumor entities, inhibition of GSK3 α/β has been demonstrated to trigger antiproliferative and proapoptotic mechanisms [12–16]. The major pathways involved in GSK3 α/β signaling are Wnt/ β -catenin, PI3K-Akt-mTOR and Ras-Raf-MEK-ERK, Hedgehog, and Notch signaling [10]. The epithelial growth factor receptor (EGFR)-PI3K-Akt signaling cascade is one of the major pathways in GEP-NETs and is often upregulated thus promoting, amongst other changes, cell cycle component-dependent tumor cell proliferation [17]. GSK3 α/β is a major downstream target of that pathway, and particularly is a direct target of Akt: activated Akt phosphorylates and inhibits GSK3 α/β [18], thereby acting to oppose the Akt-mediated proliferative changes. Moreover, GSK3 α/β may be inactivated by mTOR/p70S6K signaling, as reviewed in Maurer et al. [19].

Activated GSK3 β has also been described to negatively regulate cell cycle proteins such as cyclin D, targeting them for degradation, as reviewed in Maurer et al. [19]. On the one hand, treatment with the GSK3 inhibitors CHIR99021, 6-bromindirubin-3'-oxime-, 1-azakenpaullone, and siRNA *stimulated* proliferation of rat insulinoma INS-1E cells [20]. Conversely, the nonspecific GSK3 α/β inhibitor lithium chloride demonstrated NET growth inhibition in vitro and in vivo [21, 22]. Similarly,

in dual targeting approaches of lithium chloride and histone deacetylase inhibitors, a synergistic antiproliferative effect mediated by increased GSK3 α/β phosphorylation/inhibition and Notch1 expression was observed in gastrointestinal and pulmonary tumor cell lines [23]. Treatment with the GSK3 α/β inhibitors lithium chloride, SB415286, or kenpaullone attenuated cell proliferation in 3 different rodent insulinoma cell lines by facilitating G2/M cell cycle arrest and apoptosis [24]. Additionally, the compounds MG-132 [25], ZM336372 [26], metformin [27], and aspirin [28] have been shown to inhibit NET cell growth, possibly mediated by inhibition of GSK3 α/β . Nevertheless, a clinical phase II study in 15 patients with low-grade NETs (8 carcinoid tumors, 5 islet cell tumors) treated with the nonspecific GSK3 α/β inhibitor lithium chloride caused no objective responses, a median PFS of only 4.5 months in the treatment group, but pre- and posttreatment tumor biopsies showed no consistent GSK3 β inhibitory effects due to insufficient phosphorylation of GSK3 β in patients' serum levels [22]. Hence, preclinical and clinical studies considering more potent and specific GSK3 α/β inhibitors such as AR-A014418 need to be evaluated for possible treatment of NETs.

Biochemical studies have focused on GSK3 β inhibition. However, studies have also shown the importance of GSK3 α in drug resistance and cancer stem cells [10]. Hence, specific inhibition of both isoforms could provide new insights into GSK3 α/β signaling and effector proteins. Our aim was thus to evaluate the effects of the specific dual GSK3 α/β inhibitor AR-A014418 in a variety of human NET cell lines (BON1, H727, QGP1, GOT1) to gain novel insights into GSK3 α/β signaling in NETs, and to assess whether this pathway could be modulated therapeutically.

Materials and Methods

Cell Culture

Human pancreatic neuroendocrine BON1 tumor cell line [29], human pancreatic islet tumor cell line QGP1 [30], and human bronchopulmonary neuroendocrine NCI-H727 (H727) tumor cells [31] were obtained from Prof. R. Göke (Marburg, Germany), JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank), and ATCC (Manassas, VA, USA), respectively. Human midgut carcinoid GOT1 cells [32] were kindly provided by Prof. O. Nilsson, Sahlgrenska University Hospital Göteborg, Sweden. All cell lines used in this study were certified by the German Biological Resource Centre DSMZ (Braunschweig, Germany) by short tandem repeat typing. All human neuroendocrine cell lines were tested mycoplasma free and cultured as described previously [28, 33]. BON1 and QGP1 cells were grown in DMEM/F12 (1:1) (Life

Technologies, Karlsruhe, Germany), while NCI-H727 and GOT1 were grown in RPMI-1640 (Sigma-Aldrich, Taufkirchen, Germany), both supplemented with 10% FBS (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Life Technologies), and 0.4% amphotericin B (Biochrom) at 5% CO₂ and 37°C. The GOT1 culture medium was additionally supplemented with 5 µg/mL apo-transferrin and 0.135 IU/mL insulin.

Inhibitors

The GSK3α/β inhibitor AR-A014418, as well as lovastatin and everolimus (formerly RAD001), were acquired from Sigma-Aldrich (St. Louis, MO, USA), and 5-fluorouracil (5-FU) was obtained from Selleckchem (Munich, Germany). All 4 substances were dissolved in dimethyl-sulfoxide (DMSO; Sigma-Aldrich) and stored at -20°C.

Dose Finding

We chose the AR-A014418 (effective doses ≥5 µM) according to the dose response curves previously published by others [34–36] and according to the dose response observed in the NET cell lines used in this work. These drug doses are seemingly therapeutically relevant considering the high plasma levels of other GSK3α/β inhibitors tolerated such as lithium chloride (0.6–1.2 mM) [37]. The lovastatin concentration was chosen according to the dose response in NET cells previously published by our research group [38, 39]. Drug levels of lovastatin up to 12.4 µM were tolerated in humans without dose-limiting toxicity [40]. The 5-FU concentration was chosen according to the dose response in NET cells previously published by our research group [41].

Cell Viability Assessment

Cells were seeded and grown for 24 h in 96-well plates at densities of 1,500 (BON1), 2,000 (QGP1 and H727), and 30,000 (GOT1) cells per well. Subsequently, cells were treated with different concentrations of AR-A014418 (5–100 µM), either alone or in combination with 10 nM everolimus or 5 µM 5-FU. Metabolic activity was assessed by the “Cell Titer Blue®” cell viability assay (Promega, Madison, WI, USA) after 72 and 144 h of incubation with the different substances. Cells were incubated for 4 h with Cell Titer Blue® solution, and fluorescence was measured at 560/590 nm using a GLOMAX plate reader (Promega).

Cell Cycle Analysis by Flow Cytometric Analysis (FACS)

Cell cycle distribution was analyzed following the quick method from *Nature Protocols* “Analysis of apoptosis by propidium iodide staining and flow cytometry” [42] (BD Accuri C6 Analysis). Cells were cultured in 6-well plates for 24 h, and then the medium was replaced by fresh and antibiotics-free medium, and cells were incubated with different concentrations of AR-A014418 alone (5–60 µM) or in combination with lovastatin (20 µM AR-A014418 + 10 µM lovastatin). After 48 h, cells were washed with PBS and treated with 300 µL trypsin for 5 min at 37°C. The cells were collected, washed, and resuspended in 300 µL propidium iodide (Sigma-Aldrich), and 8 h later 20,000 events from each sample were analyzed.

Protein Extraction and Western Blotting

For Western blot experiments, cells were seeded into 10-cm plates and grown for 24 h in complete medium. Then, the medium was replaced by fresh medium, and cells were incubated with different concentrations of AR-A014418 (5 and 10 µM), either alone

or in combination with 5-FU (5 µM) or everolimus (10 nM). The incubation times were up to 96 h. Western blotting was conducted as described previously [43]. The following primary antibodies were used: pAktT (Ser473) (#4060), Akt (#2920), pERK1/2 (Thr202/Tyr204) (#4370), p4EBP1 (Ser65) (#9451), 4EBP1 (#9644), cyclin D3 (#2936), Chk1 (#2360), pEGFR (Tyr1068) (#3777), EGFR (#4267), β-catenin (#8480), pp70S6K (Thr389) (#9205), p70S6K (#2708), pS6 (Ser204/4) (#5364), S6 (#2217), pGSK3α/β (Ser21/9) (#9331), GSK3α/β (#5676) (Cell Signaling, Danvers, MA, USA), CgA (ab68271) (Abcam, Cambridge, UK), Bcl2 (610539) (Transduction Laboratories, USA), actin (A5441) (Sigma-Aldrich), ERK1/2 (06–182) (Merck-Millipore, Darmstadt, Germany).

Caspase-3/-7 Activity Assay

To measure the activity of caspase-3/-7, we used the Apo-One homogeneous caspase-3/7 assay kit (Promega). 10,000 cells were seeded per well, incubated for 72 h with respective doses of AR-A014418 (20 µM), lovastatin (10 µM), or 5-FU (5 µM), and caspase-3/7 activity was assessed according to the manufacturer's instructions.

Cell Migration Assay

BON1, QGP1, and H727 cells were seeded at densities of 120,000 (BON1) and 140,000 (QGP1 and H727) cells per chamber in culture inserts (Ibidi, Munich, Germany). After 24 h, the inserts were removed, and the cells were treated with different concentrations of AR-A014418 (5 and 20 µM). After 48 h of incubation with AR-A014418, pictures of the gap between the two cell layers were taken (Zeiss, Axiovert 135 TV [microscope] and Zeiss, AxioCam MRm [camera]) and analyzed.

γ-Irradiation Treatment

Cells were irradiated at indicated doses with an RS-225 cabinet (200 kV and 10 mA, Thoraeus filter, 1 Gy in 1 min 5 s; Xstrahl, Camberley, UK), as described [44].

Colony Formation Assays

Clonogenic survival after γ-irradiation was examined by colony formation assay. BON1, QGP1, and H727 cells were seeded as single cell suspensions into 6-well plates in a range of 200–400,000 (BON1), 300–400,000 (QGP1 and H727) or 200–200,000 (BON1, QGP1 and H727) cells per well. Cells were treated with AR-A014418 (1 or 2 µM) or DMSO as vehicle control after 4 h of adherence and incubated for 1 additional hour before being irradiated at the indicated doses. For 27 days, colony formation was allowed, before cells were stained with 80% ethanol containing 0.3% methylene blue. Amounts of 50 cells or more were considered as a colony and the percentage of surviving colonies was normalized to the plating efficiency.

Statistical Analysis

The results are displayed as mean ± standard deviation of the mean (SD) of at least 3 independent experiments. Each cell viability experiment consisted of at least 6 samples per concentration and incubation time. A priori tests considering the normal distribution and homogeneity of variances were performed applying the Kolmogorov-Smirnov test and the Levene's test of the SPSS statistical package (SPSS version 13.0 for Windows, SPSS Inc., Chicago, USA). When parametric criteria were met, an ANOVA compari-

Fig. 1. Basal protein expression level of GSK3α/β in all four NET cell lines evaluated by Western blot analysis and densitometry quantification. A representative blot out of 3 and a densitometry quantification graphic with arithmetic mean and standard deviation of at least 3 independent experiments are displayed.

son of means with a post hoc Tukey test or a two-tailed *t* test was performed; when nonparametric criteria were met, the Kruskal-Wallis test followed by the Mann-Whitney test was performed. Statistical significance was assessed at $p < 0.05$. To identify potential synergistic or antagonistic effects between AR-A014418 plus lovastatin, everolimus, or 5-FU on the natural logarithm of cell viability, we used linear mixed effects models. AR-A014418 (in 3 different doses) plus lovastatin, everolimus, or 5-FU, and the interactions between both as well as the incubation time, were considered as fixed effects, the trial as a random effect. If a significant positive interaction between AR-A014418 and the respective drug on cell survival was found, we concluded an antagonistic effect; if a significant negative interaction on cell survival was found, we concluded there was a synergistic effect. One model was estimated for each cell line. Statistical significance was defined at $p < 0.05$ using R 3.3.3 (GNU General Public License; R Foundation, Vienna, Austria). Statistically significant synergism ($p < 0.05$) was marked as “syn” in the figures.

Results

GSK3α/β Expression in NET Cells

All 4 NET cell lines investigated showed GSK3α/β expression to a variable extent (Fig. 1). GOT1 cells showed the highest GSK3α/β expression followed by QGP1 and

BON1 cells (Fig. 1). The weakest GSK3α/β expression level was detected in H727 cells (Fig. 1).

AR-A014418 Significantly Reduced NET Cell Viability *in vitro*

Neuroendocrine pancreatic (BON1), pancreatic islet (QGP1), pulmonary (H727), and midgut (GOT1) NET cells showed a significant decrease in cell viability in a time- and dose-dependent manner after AR-A014418 treatment (Fig. 2a). Cells from all 4 cell lines were incubated with AR-A014418 at concentrations from 5 to 100 μM for 144 h (Fig. 2a). The most sensitive cell line was GOT1 with an IC_{50} value of 17.9 μM followed by BON1 with an IC_{50} value of 21.7 μM and QGP1 with an IC_{50} value of 22.9 μM . The least sensitive cell line H727 showed an IC_{50} value of 23.5 μM .

AR-A014418 Inhibited GSK3α/β at Different Concentrations

All graphs of Western blot quantifications including error bars for all Western blots shown in the paper are represented as online suppl. Fig. 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000481887).

Fig. 2. Effect of AR-A014418 (AR) on cell survival of 4 different NET cell lines after 144 h of incubation (**a**) and phosphorylation of GSK3α/β after 48 h of incubation with AR (**b**). Human neuroendocrine pancreatic BON1, pancreatic islet QGP1, bronchopulmonary NCI-H727, and ileal GOT1 cells were incubated with AR (5–100 μM) for 144 h. The arithmetic means and standard deviation of at least 3 independent experiments are shown. Statistically significant different results in comparison to control DMSO are shown, considering *** $p < 0.001$. **b** Phosphorylation/inhibition of GSK3α/β (GSK3α and GSK3β) after 48 h of AR treatment. A representative blot out of 3 independently performed experiments is shown.

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GSK3α inhibition by phosphorylation was already seen at 5 μM and higher concentrations in all 4 cell lines, but additional GSK3β inhibition required higher concentrations of AR-A014418 (≥ 10 μM in GOT1 and BON1 and ≥ 20 μM in H727). In QGP1 cells, no inhibition of GSK3β was observed (Fig. 2b).

AR-A014418 Significantly Increased Sub-G1 Phase in BON1, GOT1, QGP1, and H727 Cells Indicating Cell Death, but Apoptosis Was Only Induced in GOT1, QGP1, and H727 Cells

FACS analysis showed a significant increase in the sub-G1 population for BON1, QGP1, H727, and GOT1 cells, indicating cell death (Fig. 3a; online suppl. Fig. 2).

(Figure continued on next page.)

Caspase 3/7 assay showed significant induction of apoptosis by AR-A014418 in H727 and GOT1 cells at 72 h of treatment (Fig. 3b, online suppl. Fig. 3). At 144 h of treatment caspase 3/7 assay showed significant apoptosis in H727, GOT1, and QGP1 cells, but not in BON1 cells (online suppl. Fig. 3). In BON1, LC3A expression was increased as potential evidence for autophagy (online suppl. Fig. 4). Western blot analysis showed a downregulation of antiapoptotic Bcl2 in H727 and an increase of Bcl2 in BON1 cells, but no relevant changes in QGP1 or GOT1 cells after incubation with AR-A014418 (Fig. 3c).

Downregulation of Cyclin D3 and Activation of Chk1 in BON1, H727, and QGP1 Cells, but Increase in Cyclin D3 and Inhibition of Chk1 in GOT1 Cells

Moreover, the cell cycle component cyclin D3 was downregulated in BON1, H727, and QGP1 cells, but *increased* in GOT1 cells at 20 μM AR-A014418 for 48 h; cyclin D1 was also decreased in BON1, H727, and QGP1 cells, but showed only very low expression in QGP1 and no expression in GOT1 cells, and therefore was not further investigated (online suppl. Fig. 4); the checkpoint kinase 1 (Chk1) was activated/phosphorylated after 48 h of

Fig. 3. AR-A014418 (AR)-induced increase in sub-G1 phase indicated cell death in BON1, QGP1, H727 cells (a) and GOT1 cells (online suppl. Fig. 2), but induction of apoptosis was seen in H727 cells (b, c), GOT1 cells, and QGP1 cells, but not in BON1 cells (online suppl. Fig. 3). **a** Cell cycle analysis of BON1, QGP1, and H727 cells with different concentrations of AR (5 to 60 μ M) after 48 h of incubation measured by FACS. The arithmetic means and standard deviation of at least 3 independent experiments are shown. Statistically significant different results in comparison to control DMSO are shown, considering * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

DMSO are shown, considering * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **b** Protein expression level of Bcl2 in all 4 tested cell lines measured by Western blot analysis after 48 h of incubation with AR. A representative blot out of 3 independently performed experiments is shown. **c** Caspase 3/7 activity in H727 cells after 72 h of incubation with AR. The arithmetic means and standard deviation of at least 3 independent experiments are shown. Statistically significant different results in comparison to control DMSO are shown, considering ** $p < 0.01$ and *** $p < 0.001$.

incubation in BON1, QGP1, and H727 cells with the strongest increase in H727 cells, but inhibited in GOT1 cells (Fig. 4).

AR-A014418 Caused Inhibition of EGFR and mTORC1/p70S6K Signaling, but Activation of Akt and ERK

EGFR signaling, as well as downstream components of the mTORC1 pathway, such as p70S6K, S6 and 4EBP1,

were inhibited by dephosphorylation in all 4 cell lines after 48 h of incubation with AR-A014418 (Fig. 4).

Short-term AR-A014418 treatment (2 h) led to phosphorylation/activation of Akt in all 4 lines, while ERK was only activated in BON1, GOT1, and QGP1 cells, showing strongest activation at 10 μ M AR-A014418 (Fig. 4).

As GOT1 cells showed the highest sensitivity to AR-A014418 treatment alone, further combination treatments were only conducted with the less sensitive cell

Fig. 4. AR-A014418 (AR) caused inhibition of EGFR and mTORC1-p70S6K signaling in all 4 cell lines; cyclin D3 downregulation as well as pChk1 induction was seen in BON1, QGP1, and H727 cells, but not in GOT1 cells. Western blot analysis of 4 different NET cell lines after 48 h of incubation with AR is shown. AR led to compen-

satory activation of Akt and ERK. Only H727 cells showed no ERK activation. Protein expression level of (p)Akt and (p)ERK was analyzed by Western blot after 2 h of incubation with AR. A representative blot out of 3 independently performed experiments is shown.

lines BON1, QGP1, and H727 in order to reduce the doses compared to single agent treatment, and to maximize any synergistic effects at doses which are subtherapeutic in monotherapy.

Combination Treatment of AR-A014418 plus Lovastatin Showed Synergistic Inhibitory Effects on BON1 and QGP1 Cell Viability

In order to counteract the potentially compensatory Akt and ERK activation and enhance AR-A014418 treatment effects, we investigated combination treatment with the known Akt and ERK inhibitor lovastatin (Fig. 5). Combination treatment with AR-A014418 plus lovastatin displayed significant synergistic effects on BON1 and QGP1 cell eradication after 144 h of incubation ($p < 0.05$) (Fig. 5a), and prevented AR-A014418-induced Akt and ERK activation (Fig. 5b). Although the synergistic effect in QGP1 cells seemed rather slight, this was statistically significant since AR-A014418 alone had almost no effect on cell survival, but the combination, nevertheless, slightly improved the efficacy of lovastatin alone. Clearly, it is not known whether this small effect would be relevant in vivo. This still has to be assessed.

We also tested H727 cells under the same conditions and found a significantly stronger inhibitory effect of the combination treatment compared to single substance treatment on cancer cell survival, but no synergism (online suppl. Fig. 5). The combination treatment of AR-A014418 plus everolimus showed only additive or no effects at lower concentrations in all 3 cell lines, and at high concentrations even antagonistic effects (online suppl. Fig. 6).

Combination Treatment of AR-A014418 plus 5-FU Showed Synergistic Inhibitory Effects on QGP1 Cell Viability

Combination treatment with AR-A014418 (from 2.5 to 20 μM) plus 5-FU (5 μM) for 72 h/144 h synergistically ($p < 0.01$) decreased QGP1 cell viability (Fig. 6). In H727 and BON1 cells, a significantly stronger effect of the combination treatment compared to single substance treatment in cancer cell eradication was observed, but no synergism (online suppl. Fig. 5).

AR-A014418 Significantly Inhibited Cellular Migration and Decreased β -Catenin

The migratory potential of BON1, QGP1 and H727 cells was significantly inhibited after 48 h of incubation with 20 μM of AR-A014418 compared to the control (Fig. 7a). The strongest inhibition of migration was

achieved in BON1 cells followed by H727 cells, and the lowest inhibition was seen in QGP1 cells. Downregulation of β -catenin in response to AR-A014418 treatment was observed in all 3 cell lines after 48 h of incubation (Fig. 7b). Due to an extremely long doubling time of approximately 3 weeks, GOT1 cells were not suitable for the migration assay [45].

AR-A014418 Displayed Only a Minimal Impact on the Radiosensitivity of NET Cells

We also tested the impact of GSK3 α/β inhibition on the response of NET cells to γ -irradiation. We treated BON1, QGP1, and H727 cells with γ -irradiation, either alone or in combination with AR-A014418 (1 and 2 μM). Our results reflected no radioprotection, but minimal radiosensitizing by AR-A014418 in all 3 cell lines, becoming evident only at doses of more than 5 Gy (online suppl. Fig. 7). Since GOT1 cells did not form colonies, they were not suitable for a radiosensitivity assay. Higher dose experiments could not be performed due to cell detachment.

Discussion

We evaluated the effects of the specific GSK3 α/β inhibitor AR-A014418 to gain novel insights into GSK3 α/β signaling in NETs. Specific GSK3 α/β inhibition by AR-A014418 caused a significant time- and dose-dependent decrease in NET cell survival in all cell lines tested with relevantly stronger effects after 144 h of treatment. This might be due to the relatively indolent nature of GEPNET cells and due to the fact that for GSK3 α/β inhibitors, relatively high doses are regularly required to yield an effect, and that these doses can be reduced after longer incubation times. The cell signaling changes and cellular adaptation of protein expression that was already seen at 48 h of AR-A014418 treatment might precede the viability changes seen after longer incubation times and indeed cause them. Especially growth factor receptors usually show stronger reactions after shorter incubation times. Nonspecific GSK3 α/β inhibition has already shown anti-tumor potential in various tumor entities including NETs in vitro [14, 21] and in vivo [13, 15, 22, 24]. Although IC_{50} values seemed rather high for AR-A014418, in all other in vitro studies performed, similar doses ($\geq 5 \mu\text{M}$) of AR-A014418 were necessary in order to obtain significant inhibitory effects on cell survival [34, 36, 46]. In our studies, significant effects on cell survival were also seen at 5–20 μM in all experiments, and it is worth mentioning that the

Fig. 5. Combination treatment of AR-A014418 (AR) plus lovastatin (Lova) showed a synergistic inhibitory effect on BON1 and QGP1 cell viability (**a**) through simultaneous ERK and Akt inhibition (**b**). **a** BON1 and QGP1 cells were incubated with AR (2.5–20 μ M) alone and in combination with Lova (10 μ M) for 144 h. The arithmetic means and standard deviation of at least 3 independent experiments are shown. Statistically significant different (but non-synergistic) results in comparison to each drug separately are shown as * $p < 0.05$ and *** $p < 0.001$; statistically significant syn-

ergism of the combinations is shown as “syn” ($p < 0.05$). In BON1 cells, the combination of 10 μ M Lova plus 2.5 μ M AR and the combination of 10 μ M Lova plus 20 μ M AR were statistically synergistic (syn) ($p < 0.05$ and $p < 0.0001$, respectively). In QGP1 cells, the combinations of 10 μ M Lova plus 2.5–15 μ M AR were statistically synergistic (syn) ($p < 0.05$). **b** Expression level of (p)Akt and (p)ERK in BON1 after 2 h of incubation with AR and 72 h of incubation with Lova. A representative blot out of 3 independently performed experiments is shown.

Fig. 6. Combination treatment of AR-A014418 (AR) plus 5-FU showed a statistically significant synergistic effect on QGP1 cell viability inhibition (syn) ($p < 0.01$). QGP1 cells were incubated with AR (2.5–20 μM) alone and in combination with 5-FU (5 μM) for 144 h. The arithmetic means and standard deviation of at least 3 independent experiments are shown. Statistically significant syn-

ergism of the combinations is shown as “syn” ($p < 0.01$). In QGP1 cells, the combinations of 10 μM lovastatin (Lova) plus 2.5–20 μM AR were statistically synergistic (syn) ($p < 0.01$). However, for the lowest AR dose (2.5 μM) alone, we found a positive instead of a negative effect, which makes the data for 2.5 μM AR difficult to interpret.

nonspecific GSK3 α/β inhibitor lithium chloride is clinically used at very high doses (450–1,200 mg/day) (NCT00790582) (NCT02198859) with a plasma concentration tolerability from 0.6 to 1.2 mM [37]. Due to the relatively high doses of AR-A014418 used in this study, the clinical relevance of these findings still needs to be established, although the doses might be therapeutically relevant when compared to the ready tolerability of lithium chloride. Full cellular responses to GSK3 α/β inhibition, i.e. significant effects on other signaling pathways, were obtained at concentrations at which both isoforms were inhibited: this might be explained by the fact that both molecules have distinct roles and functionalities [10]. Only in QGP1 cells GSK3 α inhibition alone was able to trigger a full cellular response, similar to a study with neuroblastoma cells where AR-A014418 showed growth inhibition after sole GSK α phosphorylation at concentrations up to 20 μM [35].

FACS analysis implicated significant induction of cell death in all 4 NET cell lines investigated. However, only in H727, GOT1, and QGP1 cells was there a significant induction of apoptosis with increased caspase 3/7 activity. In H727 cells, we additionally observed decreased antiapoptotic Bcl2 expression after AR-A014418 treatment. Similar results regarding apoptosis induction and a decrease in Bcl2 after GSK3 α/β inhibition were described

recently for different cancer cell entities in vitro [12, 16, 24] including NETs [21]. Furthermore, neuroblastoma cell growth inhibition after treatment with AR-A014418 was due to apoptosis induction [35]. However, in BON1 cells even a decrease in cleaved caspase 3/7 at 72 h and 144 h of treatment and an increase in antiapoptotic Bcl2 were observed. Since decreased apoptosis might indicate an increase in autophagy, we also investigated the autophagy marker LC3A in BON1 cells, and found increased expression of LC3A, indicating autophagy in BON1 cells. Hence, we conclude that the remaining possible cellular death mechanism in BON1 cells might include autophagy or necrosis potentially due to mitotic catastrophe, as described before for GSK3 α/β inhibition in cells not undergoing apoptosis [34]. Further studies are necessary to prove these cell death mechanisms in response to GSK3 α/β inhibition in BON-1 cells.

Moreover, we found evidence for autophagy in BON1 cells, as indicated by increased cleavage of LC3A.

In all tested NET cell lines expressing EGFR, AR-A014418 inhibited EGFR signaling. The effect of GSK3 α/β on the EGFR does not seem to be a direct effect. The EGFR signaling pathway may be activated alternatively via NF- κB . Since GSK3 may activate NF κB , inhibition of GSK3 leads to inhibition of NF- κB . Therefore, the EGFR inhibition in response to GSK3 inhibition might be due

Fig. 7. AR-A014418 (AR) significantly inhibited cellular migration (**a**) and downregulated β -catenin (**b**). **a** Cellular migration potential of BON1, QGP1, and H727 cells was tested. The arithmetic means and standard deviation of at least 3 independent experiments are shown. Statistically significant different results in com-

parison to control DMSO are shown, considering * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **b** The expression level of β -catenin was evaluated via Western blot analysis after 48 h of incubation with AR. A representative blot out of 3 independently performed experiments is shown.

to inhibition of NF κ B, as reviewed in Maurer et al. [19] and Mills et al. [47]. In NETs, overexpression and activation of EGFR has been shown to lead to proliferative and mitotic signaling cascades [17, 48]. Unfortunately, EGFR inhibition by gefitinib in patients with midgut NETs has shown only a poor response [49]. Nevertheless, EGFR remains a rational target, but current drugs might not adequately inhibit this receptor [50]. Therefore, EGFR inhibition by AR-A014418 might contribute to its antitumor potential in NETs and be an interesting novel aspect of this drug.

One of the major growth factor-induced signaling pathways, which often bears activating mutations and

shows overexpression in NETs, is the PI3K/Akt/mTORC1 pathway [17, 51]. We found that AR-A014418 efficiently dephosphorylated/inhibited key effector proteins of the mTORC1/p70S6K pathway, such as p70S6K, 4EBP1, and S6 in NETs. Similar effects on mTORC1, 4EBP1, and S6 by AR-A014418 were observed in a study with renal cell carcinoma [36]. Interestingly, Ito et al. [36] found that GSK3 regulated the phosphorylation of 4EBP1 directly and S6 independent of PI3K/Akt/mTORC1 signaling. The PI3K-Akt-mTOR pathway is also a crucial molecular signaling pathway to promote cell cycle component-dependent proliferation, as it converges on the cell cycle [52]. As a possible consequence of mTORC1/p70S6K in-

hibition, the cell cycle components cyclin D1 and cyclin D3 were also decreased with AR-A014418 treatment in BON1, QGP1, and H727, but not in the most sensitive GOT1 cells, according to our results. Due to a previously reported negative regulation of cell cycle proteins by active GSK3 β [19], we would have expected an increase in cyclin D1 and D3 in response to GSK3 β inhibition, which could not be confirmed in the less sensitive cell lines BON1, QGP1, and H727. In neuroblastoma cells, a decrease in the related cyclin D1 was also observed after AR-A014418 treatment [35]. In contrast, in the most sensitive GOT1 cells, there was no expression of cyclin D1 and an increase in cyclin D3 at 20 μ M AR-A014418. This increase in cyclin D3 might be a direct effect of strong GSK3 β inhibition, since active GSK3 β has been described to lead to degradation of cell cycle proteins such as cyclin D [19]. Therefore, strong GSK3 β inhibition would be expected to result in an increase in cyclin D3, as found in GOT1 cells. One of the best-known substrates of cyclin D/Cdk4 and -6 is the retinoblastoma tumor suppressor protein (Rb). If nonphosphorylated, Rb binds to E2F and prevents expression of E2F-regulated genes (amongst others cyclin F). The cyclin D/Cdk4 and -6 complex inhibits Rb by partial phosphorylation, reducing its binding to E2F and thereby allowing E2F-mediated transcription of genes and thus cell cycle progression. In a previous study on GOT1 cells, as compared to other NET cell lines, we reported a poorly detectable expression level of Rb [41] likely to facilitate, together with cyclin D3 upregulation in response to GSK3 α/β inhibition, cell cycle progression and apoptosis induction [53, 54]. However, neither in GOT1 cell lines with low Rb expression and increased cyclin D3 expression nor in the other 3 NET cell lines with wild-type Rb and reduced cyclin D1 and D3 levels in response to GSK3 inhibition relevant changes in the cell cycle activity were found.

Activation/phosphorylation of Chk1 was found to be increased in BON1, QGP1, and H727 cells after AR-A014418 treatment, suggesting an intent to restore DNA integrity and to prevent damaged cells from cell cycle progression by activating the DNA damage response [55]. The absent Chk1 phosphorylation/activation in GOT1 cells upon AR-A014418 treatment might contribute to the higher sensitivity to AR-A014418 treatment. Effects on Chk1 phosphorylation might not be a direct consequence of GSK3 α/β inhibition, but an indirect response of the stressed and dying cells after AR-A014418 treatment.

However, we found a compensatory activation of Akt and ERK signaling in response to AR-A014418 treatment

that potentially counteracts the antiproliferative effects of AR-A014418. This potential escape mechanism of the cell may be due to a similar mechanism as caused by the mTORC1-inhibitor everolimus that also leads to compensatory Akt and ERK activation [56–60]. Compensatory feedback loops of PI3K/Akt/mTORC1 and Ras/Raf/MEK/ERK signaling are well described in NET cells [17, 61–63]. Therefore, we tested a combination treatment with the HMG-CoA reductase inhibitor, lovastatin, which is known to inhibit Akt and ERK signaling and has demonstrated antitumor potential in many cancer types including NETs in vitro [38, 39, 64, 65]. The combination treatment with AR-A014418 plus lovastatin indeed showed synergistic growth inhibition in BON1 and QGP1 and additive effects in H727 cells. Accordingly, we could show that lovastatin prevented Akt and ERK activation induced by AR-A014418 alone. Similarly, our previous studies have shown that lovastatin prevented ERK activation induced by PI3K/mTORC1/2 inhibition in pheochromocytoma cells [66] and Akt activation induced by mTORC1 inhibition in NETs [38].

The combination treatment of AR-A014418 plus everolimus showed only additive or no effects at lower concentrations, and at high concentrations even antagonistic effects. Similar antagonizing effects of simultaneous mTORC1 inhibition and GSK3 α/β inhibition were obtained in a study with renal cell carcinoma, and were explained by a competitive inhibition of 4EBP1 and S6, as GSK3 α/β and mTORC1 share 4EBP1 and S6 as target substrates [36].

5-FU, a standard chemotherapy in combination with streptozotocin or temozolomide for pancreatic NETs according to current guidelines [67], also showed significant synergistic effects in combination with AR-A014418 in QGP1 and additive effects in BON1 and H727 cells. Hence, treatment with AR-A014418 displayed promising chemosensitizing properties in NET cell lines. In previous studies, GSK3 α/β overexpression correlated with chemoresistance [68, 69], and the combination of AR-A014418 with the chemoactive agents gemcitabine or docetaxel also displayed synergistic effects on pancreatic adenocarcinoma or renal cancer cell death in vitro and in vivo [16, 46]. Therefore, the overexpression of GSK3 α/β observed in GOT1 cells compared to the other NET cell lines might contribute to the chemoresistance of well-differentiated ileal NETs [70] if this is translatable to patient tumor tissues.

GSK3 α/β has been shown to play a crucial albeit ambiguous role in cellular migration [71]. We could also show significant inhibition of cellular migration after

AR-A014418 treatment in all 3 cell lines tested. However, for QGP1 cells, the decrease in migration potential was rather moderate, and thus the clinical relevance remains unclear. Furthermore, the inhibition of cellular migration in NET cells after AR-A014418 treatment was associated with decreased β -catenin expression. Similarly, accumulation of β -catenin has been associated with an enhanced migratory potential, affecting migration-promoting factors [72, 73]. Furthermore, reduction of β -catenin expression after AR-A014418 treatment was also observed in neuroblastoma cells [35]. This might be explained by the fact that GSK3 β has been shown to be implicated not only in the degradation of β -catenin, but also in the stabilization of β -catenin through specific complex phosphorylation [10]. Hence, GSK3 α/β inhibition might lead to β -catenin destabilization/degradation in this context.

GSK3 α/β downregulation has been shown to play a role in the radioresistance of tumor cells through activation of Akt signaling and cyclin D1 [74]. We also found Akt activation in response to GSK3 α/β inhibition in all 3 NET cell lines tested. Therefore, we evaluated the effects of AR-A014418 on clonogenic cell growth upon γ -irradiation treatment. However, we found that GSK3 α/β inhibition did not lead to radioresistance, but even had a minimal radiosensitizing effect in BON1 and QGP1 cells. In a previous study with pancreatic adenocarcinoma cell lines, the GSK3 α/β inhibitor AR-A014418 also displayed radiosensitizing characteristics upon ionizing radiation [46]. These results indicate that GSK3 α/β inhibition by AR-A014418 most likely would not interfere with the efficacy of radiotherapeutic intervention for NETs and may even enhance its effects. Finally, it is worth mentioning that the cell lines used are not completely representative of most NETs, since whole-exome comparison of primary well-differentiated NETs and their cell lines, which included pancreatic BON1, QGP1, and bronchopulmonary NCI-H727 cells, have shown differences in mutation-rate and -pattern of tested NET cell lines from well-differentiated NETs in patients [75, 76]. The Rb status in BON1, QGP1, and H727 cells is likely to reflect wild-type conditions. However, in another study, we reported that GOT1 cells showed only poorly detectable expression level of Rb [41]. This is in contrast to the “real life” situation, since Rb is highly expressed in various NET types taken from patients [77–79]. The mTOR mutation status has been described in whole-exome studies conducted recently, and no mTORC1/2 mutations have been found [75, 76], but there are no sequencing data especially of the mTOR status available in the cell lines used for our work. We ac-

cept that the available human NET cell lines might not adequately reflect the genetic and biological make-up of well-differentiated NETs, but currently they are the principal research tool we can use. Nevertheless, in order to incorporate our results into a more translatable study, we recommend *in vivo* studies to investigate the efficacy of AR-A014418 in NETs, but the use of these cell lines is a necessary first step.

Conclusions

The specific GSK3 α/β inhibitor AR-A014418 significantly decreased NET cell viability in a time- and dose-dependent manner through inhibition of EGFR and mTORC1/p70S6K signaling. Compensatory Akt and ERK activation in response to GSK3 α/β inhibition was reversed by combination treatment with lovastatin, which was synergistic in BON1 and QGP1 cells. In addition, AR-A014418 showed significant chemosensitizing effects on 5-FU in QGP1 cells. Moreover, cellular migration was significantly negatively regulated by GSK3 α/β inhibition possibly through β -catenin downregulation. Taken together, inhibition of GSK3 α/β by specific inhibitors such as AR-A014418 might be an interesting novel approach in the treatment of NETs, and the therapeutic effect might even be synergistically enhanced in combination with already approved drugs such as lovastatin or 5-FU. Clinical phase 1/2 studies with novel specific GSK3 α/β inhibitors in the treatment of NETs are warranted.

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P.G. has received research contracts (Ipsen, Novartis) and lecture honoraria (Ipsen, Novartis). A.B.G. has received lecture fees and advisory board honoraria from Novartis. C.J.A. has received research contracts (Ipsen, Novartis), lecture honoraria (Ipsen, Novartis, Pfizer, Amgen, Roche, Falk), and advisory board honoraria (Novartis). S.N. has received a research contract (Novartis), but not for this current project, and lecture honoraria (Ipsen). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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