

**Research paper****Anticancer effects of metformin on neuroendocrine tumor cells *in vitro***

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**ABSTRACT**

**Metformin is a widely used oral antidiabetic drug with good tolerability. Recent studies suggest that it also possesses adjuvant potent anticancer properties in a variety of tumors. Neuroendocrine tumors (NETs) of the gastro-entero-pancreatic system (GEP) comprise a heterogeneous group of tumors with increasing incidence and limited effective therapeutic options. Here we report the antiproliferative effects of metformin in neuroendocrine tumor cells *in vitro*. Treatment of human pancreatic BON1, bronchopulmonary NCI-H727, and midgut GOT1 neuroendocrine tumor cells with increasing concentrations of metformin (0.1-10 mM) dose-dependently suppressed cell viability and cell counts. Metformin induced AMPK phosphorylation in pancreatic BON1 and midgut GOT1 but suppressed AMPK activity in bronchopulmonary NCI-H727. Thus, AMPK-dependent and AMPK-independent properties may be operative in NETs of different origin. Metformin suppressed mTORC1 signaling in all three tumor cell types, evidenced by suppression of 4EBP1, pP70S6K, and S6 phosphorylation, and was associated with compensatory AKT activity. We observed induction of ERK phosphorylation in BON1 and NCI-H727 and inhibition of ERK in midgut GOT1 cells, while all three tumor cell types responded with induction of GSK3 phosphorylation. This suggests a central role for GSK3 in metformin-mediated signal transduction. Inhibition of cell proliferation by metformin was associated with apoptosis induction only in midgut GOT1, evidenced by increased subG0/1 fraction and PARP cleavage. These results suggest a potential role of metformin as a (adjuvant) therapeutic for patients with NETs.**

**Key words:** AMPK, Cell viability, Metformin, mTOR, Neuroendocrine tumor

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## INTRODUCTION

Metformin (1.1-dimethylbiguanide hydrochloride) is the most frequently used antidiabetic medication with ca. 120 million prescriptions filled every year worldwide.<sup>1</sup> This biguanide indirectly stimulates AMP-activated protein kinase (AMPK)<sup>2</sup> with subsequent reduction of hepatic gluconeogenesis and glycogenolysis and an increase in glucose uptake in the muscle.<sup>3</sup> AMPK activation also leads to suppression of the mammalian target of rapamycin (mTOR),<sup>4</sup> a key regulator of cell proliferation in cancer cells. AMPK-independent mechanisms of action of metformin include induction of cell cycle arrest and reduction of insulin/IGF-1 signaling.<sup>4,5</sup>

Over the past few years, several epidemiological and preclinical/clinical studies suggest potent anticancer effects of metformin.<sup>4,5</sup> Patients with diabetes mellitus type 2 treated with metformin appear to have a decreased risk for cancer development of the pancreas,<sup>6-8</sup> liver,<sup>9-11</sup> lung,<sup>12</sup> and breast.<sup>13</sup> Additionally, in vitro studies have demonstrated inhibitory effects of metformin on cell proliferation in endometrial,<sup>14</sup> ovarian,<sup>15</sup> glioma,<sup>16</sup> pancreatic cancer,<sup>17</sup> and melanoma<sup>18</sup> tumor cells. Thus, in addition to diabetes therapy, metformin may offer valuable anticancer effects, especially in endocrine-related cancers.<sup>19</sup>

Neuroendocrine tumors (NETs) of the gastroenteropancreatic (GEP) system arise from diffuse neuroendocrine cells within the digestive system. Although considered rare, accounting only for ~2% of all gastrointestinal tumors,<sup>20,21</sup> their prevalence has increased over the last two decades.<sup>20,21</sup> Current systemic treatment options include biotherapy with somatostatin analogues and interferon- $\alpha$ , molecular targeted therapy with everolimus and sunitinib, cytotoxic chemotherapy protocols, and peptide receptor-targeted therapy.<sup>22</sup> However, most available therapies are efficacious for a limited period of time and rarely induce tumor remission.<sup>22</sup> Despite the introduction of target-directed therapies such as the multiple tyrosine kinase inhibitor sunitinib<sup>23</sup> and the mTOR inhibitor everolimus<sup>24,25</sup> for a subset of progressive NET,<sup>22,26</sup> there is a need for novel drugs for syndromic and tumor growth control for this heterogeneous tumor entity. As inhibition of mTORC1 signaling by everolimus has already proven to be a promising target for antitumoral therapy in NETs<sup>23-25,27</sup> and metformin

also inhibits mTORC1 signaling,<sup>28-30</sup> we investigated potential effects of metformin on NETs.

## MATERIALS & METHODS

### *Materials*

DMEM/F12 media, penicillin, and streptomycin were purchased from Gibco/Invitrogen (Karlsruhe, Germany) and RPMI media was obtained from PAA (Pasching, Austria). Fetal bovine serum (FBS) and amphotericin B were acquired from Biochrom (Berlin, Germany). Metformin was purchased from Sigma-Aldrich (St. Louis, USA), IGF1 from Amersham Pharmacia Biotech Europe, and rapamycin from Biomol (Hamburg, Germany).

### *Cell cultures*

All human neuroendocrine cell lines were received and cultured as recently described.<sup>31</sup> Briefly, pancreatic neuroendocrine BON1 tumor cells<sup>32</sup> (kindly provided by R. Göke, Marburg) were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS, 10 mg/ml penicillin/streptomycin and 4 mg/ml amphotericin B. Human midgut carcinoid GOT1 cells<sup>33</sup> (kindly provided by Prof. Ola Nilsson, Sahlgrenska University Hospital Göteborg, Sweden) and human bronchopulmonary neuroendocrine NCI-H727 tumor cells (purchased from ATCC, Manassas, VA) were both cultured in RPMI medium supplemented with 10% FBS, 10 mg/ml penicillin/streptomycin and 4 mg/ml amphotericin B. Additional supplements in GOT1 culture medium were 0.135 IU/ml insulin and 5mg/dl apo-transferrin.

### *Assessment of cell viability/cell numbers*

Cell viability was assessed as recently described.<sup>31</sup> Briefly, cells were seeded into 96-well plates at densities of 3000 (BON1), 50000 (GOT1) and 4000 (NCI-H727) cells per well, respectively, and grown for 24 hrs. The next day, the medium was replaced by serum rich medium (10% FBS) containing various concentrations of metformin (0.1 to 10 mM) and the cells were further incubated for the indicated time intervals. Cell viability expressed by metabolic activity was measured with Cell Titer 96 aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Following 3 hrs of incubation with Cell Titer 96 solu-

tion, absorbance at 492 nm was determined using an ELISA plate reader (Orion II, Berthold Detection Systems, Pforzheim, Germany). Cell counts were performed with an automated cell counter (Countess™, Invitrogen, Germany).

### **Cell cycle analysis**

Cell cycle distribution was analyzed using propidium iodide staining and flow cytometry as described previously.<sup>31</sup> Briefly, cells were washed with PBS and treated with 400 ml trypsin at 37°C for 4 minutes. After another wash cycle with PBS, the cells were resuspended in 350 µl propidium iodide. Sub-G1 events and cell cycle distribution were measured in a fluorescence-activated cell sorter (BD Accuri C6 Analysis). Nuclei to the left of the G1-peak containing hypodiploid DNA were considered apoptotic.

### **Protein Extraction and Western Blotting**

Protein extraction and Western blotting were performed as described previously.<sup>31</sup> Briefly, cells were lysed in 500 µl lysis buffer (MPER® Mammalian Protein Extraction Reagent; #78501F, containing Halt™ Protease & Phosphatase Inhibitor Cocktail; #78447; PIERCE, Rockford, USA). The lysates were centrifuged for 10 min at 4°C and 13,000g and supernatants were adjusted to equal protein loads and diluted 1:1 with SDS sample buffer. Samples were boiled for 5 min and separated on a SDS polyacrylamide gel. Proteins were electrotransferred for 60 min onto PVDF membranes (Immobilone; Millipore, Eschborn, Germany) using a semi-dry Western-blot technique. After blocking in 2% non-fat dried milk, the membranes were incubated overnight in appropriate dilutions of antibodies against pAkt (Ser 473) (#4060), Akt (#2920), pERK (Thr202/Tyr204) 1/2 (#4370), pP70S6K (Thr389) (#9234), P70S6K (#9202), p4EBP1 (Ser65) (#9451), 4EBP1 (#9644), pS6 (Ser235/6 (#4858) and Ser240/4 (#5364)), S6 (#2317), pGSK3 (Ser21/9) (#9331), GSK3 (#9315), IGF1R (#3027), EGFR (#4267), PARP (#9542) (all from Cell Signaling, Danvers, MA), Erk 1/2 (06-182; Millipore). After washing with PBS, the membranes were incubated with a peroxidase-conjugated secondary antibody (1:25,000) for 2 hrs (anti-rabbit IgG; #7074 and anti-mouse IgG; #7076; Cell Signaling, Danvers, USA). The blots were washed and immersed in the chemiluminescent substrate SuperSignal West

Dura (Thermo Scientific, Rockford, USA) and exposed to Super RX X-ray film (FUJIFILM Corporation, Tokyo, Japan).

### **Statistical analysis**

For the statistical analyses the “Statistical Program for the Social Sciences-SPSS” software (v.16.0) was used. Results are expressed as mean ± SD of independently performed experiments. For proliferation assays and cell cycle analyses, comparisons were evaluated using the 2-tailed Student’s t test. Statistical significance was set at  $p < 0.05$ , after a Bonferroni adjustment to compensate for the multiple comparisons.

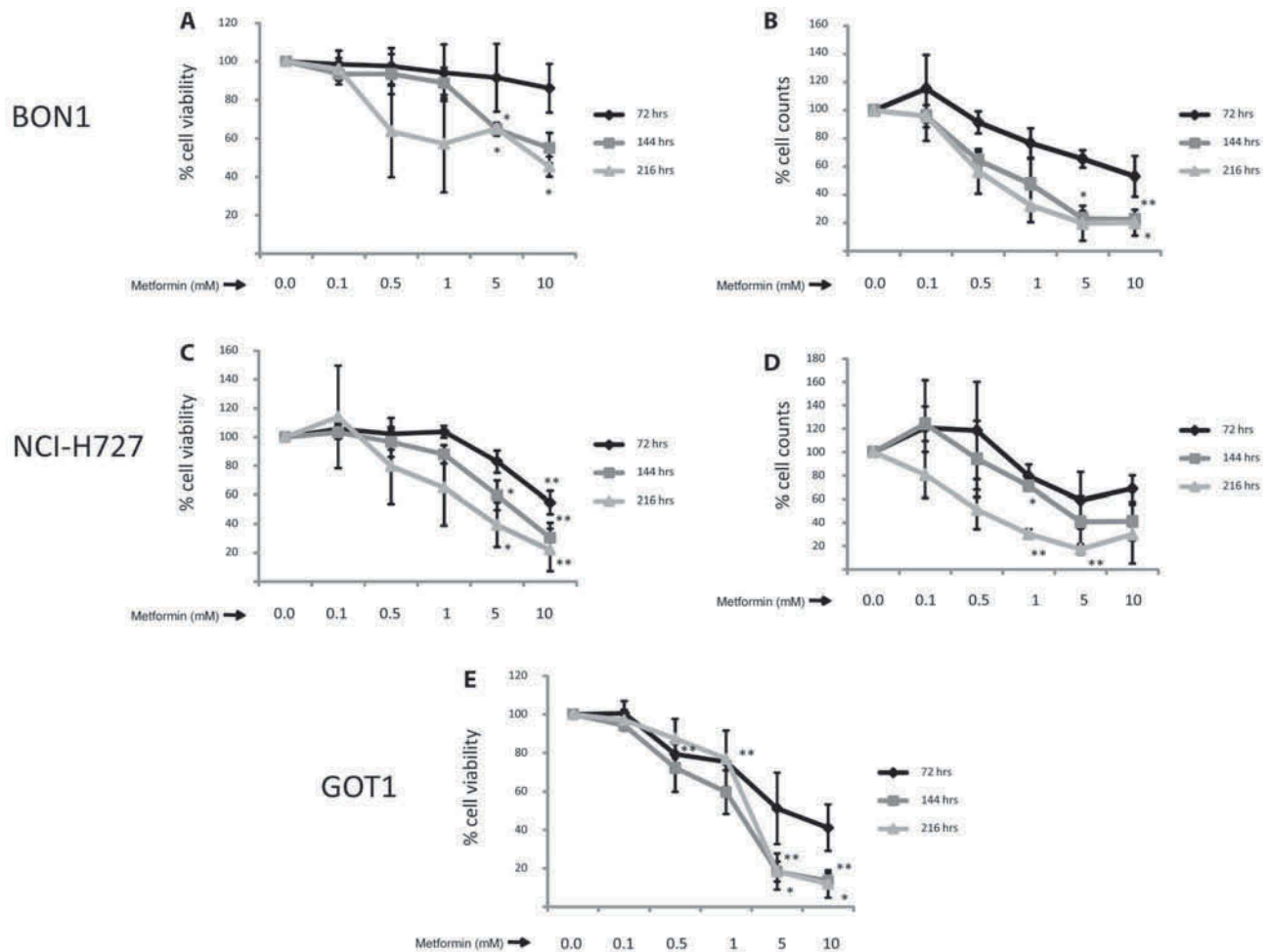
## **RESULTS**

### **Metformin inhibits neuroendocrine cell viability**

Treatment of human pancreatic neuroendocrine BON1 cells with metformin suppressed cell viability (Cell Titer Assay; Promega) in a dose-dependent manner (Figure 1A). Significant effects were observed at 144 and 216 hrs; at 144 hrs, treatment with metformin at 5 mM suppressed cell viability to  $65 \pm 3\%$  ( $p=0.015$ ) and at 10 mM to  $55 \pm 8\%$  (n.s.). At 216 hrs, treatment at 5 mM suppressed cell viability to  $65 \pm 3\%$  ( $p=0.010$ ) and at 10 mM to  $45 \pm 5\%$  ( $p=0.015$ ). Cell counts performed with an automated cell counter revealed similar results (Figure 1B); significant effects were observed at 144 and 216 hrs; maximum suppression (to ~20% at 144 and 216 hrs) was observed at the highest metformin doses tested (5 to 10 mM).

Metformin also suppressed cell viability in human bronchopulmonary NCI-H727 cells in a dose-dependent manner (Figure 1C). Significant effects were observed at all time points tested (at 5-10 mM metformin). Maximal suppression levels were observed at 10 mM metformin with inhibition of cell viability to  $55 \pm 8\%$  ( $p=0.007$ ),  $30 \pm 10\%$  ( $p=0.004$ ) and  $22 \pm 15\%$  ( $p=0.009$ ) at 72, 144 and 216 hrs, respectively. Cell counts performed with an automated cell counter demonstrated similar results (Figure 1D).

Human midgut neuroendocrine GOT1 cells demonstrated high sensitivity to treatment with metformin (Figure 1E). Significant effects were observed at starting doses of 0.5 mM (at 72 hrs) and a dose-dependent decrease in cell viability was observed for all time



**Figure 1.** Inhibition of neuroendocrine tumor cell viability by metformin. Human pancreatic BON1 (A, B), bronchopulmonary NCI-H727 (C, D) and midgut GOT1 (E) neuroendocrine tumor cells were treated with indicated concentrations of metformin for 72, 144 and 216 hrs, respectively. Cell viability based on metabolic activity was measured with Cell Titer 96 kit (Promega) (A, C and E) and cell counts were performed with an automated cell counter (B and D). Shown are the mean values  $\pm$  SD of 3 independently performed experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  versus untreated control.

points with a maximum suppression to  $\sim 10$ -20%.

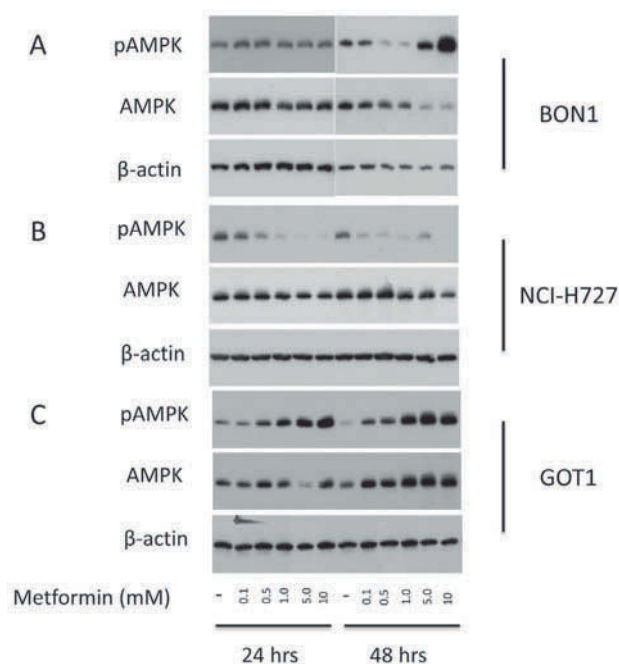
#### ***Effects of metformin on AMPK phosphorylation in neuroendocrine tumor cells***

Metformin is known to act via AMPK-dependent and -independent pathways.<sup>29,30,34</sup> Treatment of human neuroendocrine tumor cells with metformin had different effects on AMPK activity depending on the cell line used. In human pancreatic BON1 cells, treatment with 5-10 mM metformin for 48 hrs induced AMPK phosphorylation (Figure 2A). Midgut neuroendocrine GOT1 cells were highly responsive to metformin in terms of AMPK activation (Figure 2C); metformin doses as low as 0.1 mM induced AMPK

phosphorylation and positive effects were observed at all time points tested (including 2 hrs and 72 hrs, not shown). In contrast, bronchopulmonary NCI-H727 neuroendocrine cells responded with suppression of baseline AMPK phosphorylation after treatment with metformin (Figure 2B).

#### ***Effects of metformin on the PI3K/AKT/mTOR signaling pathway***

Treatment of human pancreatic neuroendocrine BON1 cells with metformin, particularly at 48 hrs, dose-dependently induced AKT phosphorylation (Figure 3A). Inhibition of the mTOR signaling pathway was confirmed by metformin-mediated suppression

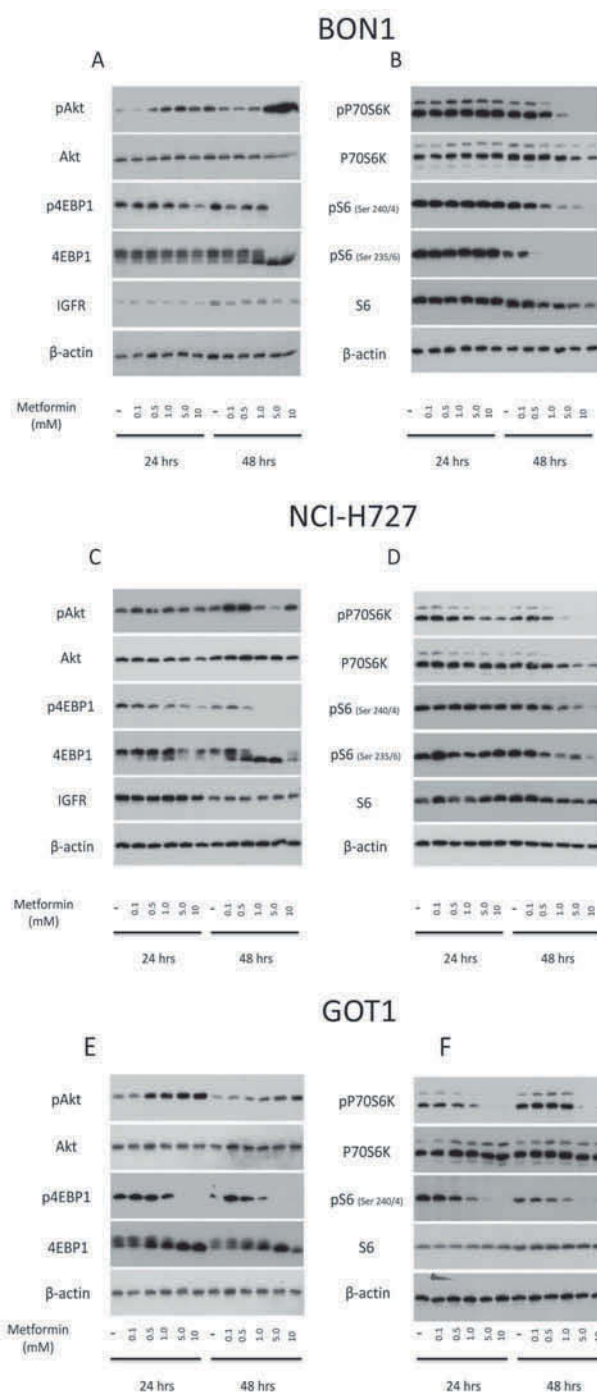


**Figure 2.** Effect of metformin on AMPK phosphorylation in neuroendocrine tumor cells. Human pancreatic neuroendocrine BON1 (upper panel; A), bronchopulmonary NCI-H727 (middle panel; B) and midgut GOT1 (lower panel; C) cells were treated with indicated concentrations of metformin for 24 and 48 hrs. Subsequently the expression of pAMPK, AMPK and  $\beta$ -actin loading control was evaluated by Western blot analysis. A representative blot out of 3 independently performed experiments is shown.

of p4EBP1 (Figure 3A), pP70S6K and S6 phosphorylation (Figure 3B). Metformin potently suppressed 4EBP1 and S6 (at Ser 235/6) phosphorylation at doses as low as 0.5 mM (Figure 3A and B), while only minor inhibitory effects on IGFR protein expression were observed.

In bronchopulmonary NCI-H727 neuroendocrine cells, metformin suppressed AKT phosphorylation at higher doses (1-10 mM), while at lower doses (0.1-0.5 mM) AKT phosphorylation was increased (at 48 hrs, Figure 3C). At starting doses of 0.5 to 1.0 mM, metformin suppressed phosphorylation of mTOR downstream targets 4EBP1, P70S6K and S6 (Figure 3C and D). No major effect was observed on IGFR protein expression.

Midgut neuroendocrine GOT1 cells (particularly at 48 hrs) also responded with decreased phosphorylation of mTOR downstream signaling targets 4EBP1



**Figure 3.** Effect of metformin on PI3K/AKT/mTORC1 signaling in neuroendocrine. Human pancreatic BON1 (A, B), bronchopulmonary NCI-H727 (C, D) and midgut GOT1 (E, F) neuroendocrine tumor cells were treated with indicated concentrations of metformin for 24 and 48 hrs, respectively. Subsequently the expression of pAKT, AKT, pEBP1, EBP1, IGFR (A, C and E) as well as pP70S6K, P70S6K, pS6, S6 (B, D and F) and  $\beta$ -actin loading control was evaluated by Western blot analysis. A representative blot out of 3 independently performed experiments is shown.

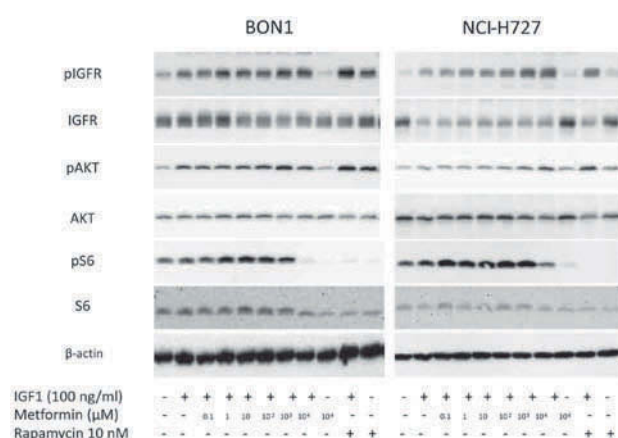


(Figure 3E), P70S6K and S6 (Figure 3F). Similar to BON1 cells, metformin dose-dependently increased AKT phosphorylation at 24 and 48 hrs (Figure 3E).

Specific treatment of neuroendocrine BON1 and NCI-H727 cells under serum-free conditions with IGF1 induced rapid (not shown) and sustained phosphorylation of the IGF receptor, as well as AKT (particularly in BON1 cells) and downstream mTOR S6 phosphorylation (Figure 4). In a dose-response curve employing a wide spectrum of metformin doses, even high metformin concentrations had no effect on IGF1-induced IGFR phosphorylation in both cell lines tested. In contrast to cells cultured in complete medium (Figure 3), under serum-free conditions (Figure 4), we did not observe major effects of metformin on basal or IGF1-induced AKT phosphorylation, but metformin dose-dependently suppressed downstream mTOR signaling, evidenced by inhibition of basal and IGF1-induced S6 phosphorylation. This inhibition of basal and IGF1-induced S6 phosphorylation by metformin was similar to inhibition of S6 phosphorylation by the established mTOR inhibitor rapamycin, used as a positive control (Figure 4).

#### ***Effects of metformin on GSK3 and ERK signaling***

All three neuroendocrine tumor cell lines re-



**Figure 4.** Effect of metformin and rapamycin on IGF1-induced signaling in neuroendocrine tumor cells. Human pancreatic BON1 (left panel) and bronchopulmonary NCI-H727 (right panel) neuroendocrine tumor cells were pretreated with indicated concentrations of metformin or rapamycin for 1 hr prior to induction with IGF1 for 24 hrs. The expression of pIGFR, IGFR, pAKT, AKT, pS6, S6 and  $\beta$ -actin loading control was evaluated by Western blot analysis. A representative blot out of 3 independently performed experiments is shown.

sponded with a dose-dependent increase in GSK3 phosphorylation (at 24 and 48 hrs) at the lowest metformin doses tested (0.1 to 0.5 mM; Figure 5A-C). The effect of metformin on ERK phosphorylation differed between neuroendocrine cells of different origin. In pancreatic BON1 and bronchopulmonary NCI-H727 cells, metformin potently induced ERK phosphorylation peaking at 1 to 5 mM (Figure 5A-B). In contrast, GOT1 cells responded with a dose-dependent suppression of ERK phosphorylation in response to low starting metformin doses (particularly at 48 hrs, Figure 5C).

#### ***Effects of metformin on apoptosis in neuroendocrine tumor cells***

In order to explore mechanisms for metformin-mediated inhibition of cell proliferation, we determined the effect of metformin on sub-G0/1 distribution in neuroendocrine tumor cells. Treatment with metformin did not increase the percentage of BON1 and NCI-H727 cells in sub-G0/1 (data not shown). Failure of apoptosis induction by metformin in these cells was confirmed by grossly unaffected levels of cleaved PARP (not shown).

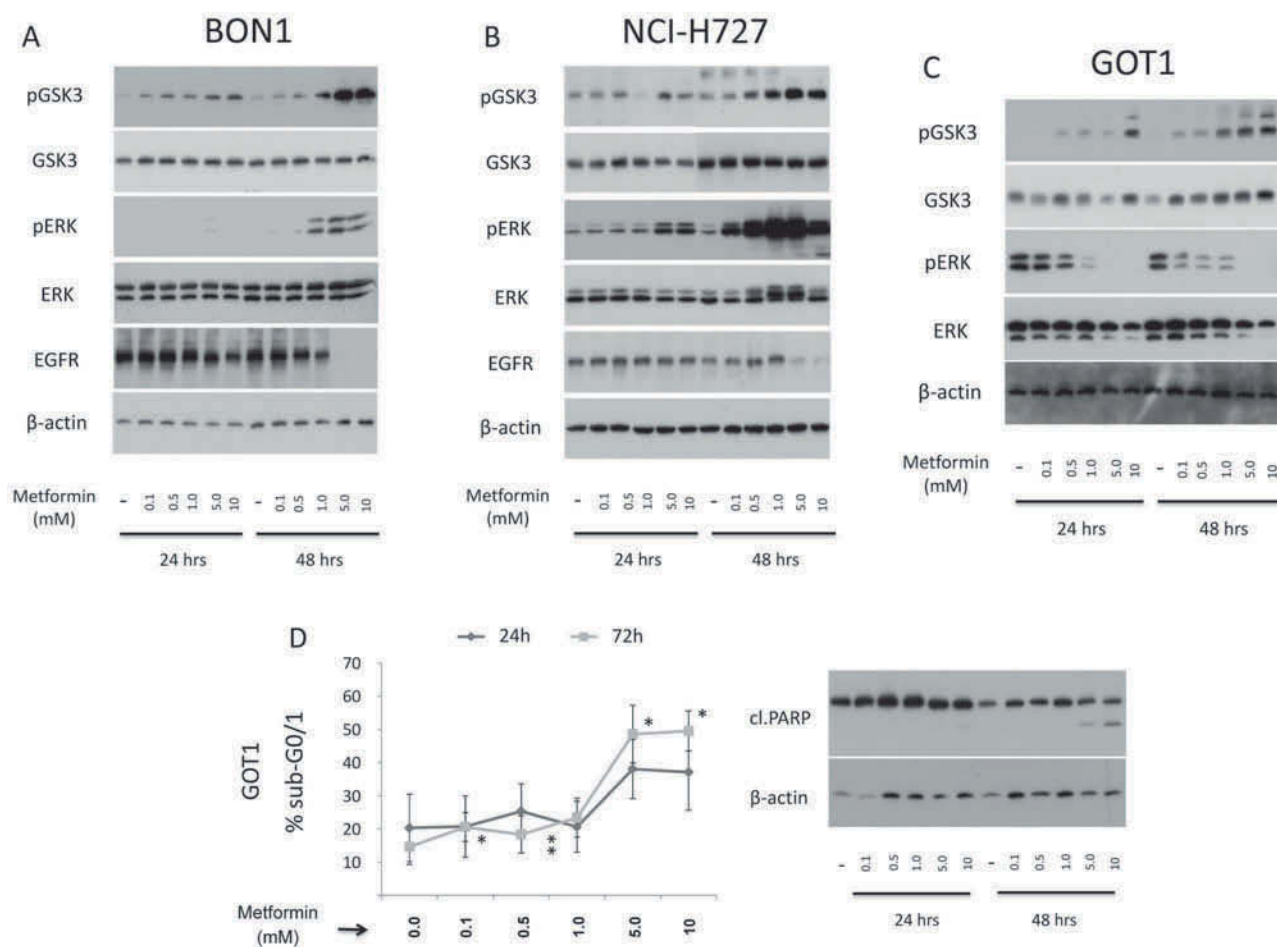
In contrast to BON1 and NCI-H727 cells, in midgut neuroendocrine GOT1 cells metformin induced apoptosis, as demonstrated by increased percentage of cells in sub-G0/1 (Figure 5D, left panel) and induction of PARP cleavage (particularly at 48 hrs, Figure 5D, right panel).

#### ***Effects of metformin on cell cycle distribution in neuroendocrine tumor cells***

Treatment of unsynchronized (culture in complete medium) pancreatic neuroendocrine BON1 cells with metformin doses 1 mM for 72 hrs reduced the percentage of cells in S phase from 16% to 10% ( $p=0.013$ ) and increased cells in G0/1 phase from 57% to 65% ( $p=0.013$  at 0.5 mM) (Figure 6A).

Similarly to the observed effects with BON1 cells, metformin dose-dependently decreased NCI-H727 cell proliferation, evidenced by decreased entry into S phase (from 13% to min. 8% at 1 mM;  $p=0.001$ ) and G2/M phase (from 27% to 20% at 10 mM,  $p<0.002$ ) and increased percentage of cells in G0/1 (from 51% to 63% at 5mM,  $p<0.006$ ) at 72 hrs (Figure 6B).

In contrast, midgut neuroendocrine GOT1 cells



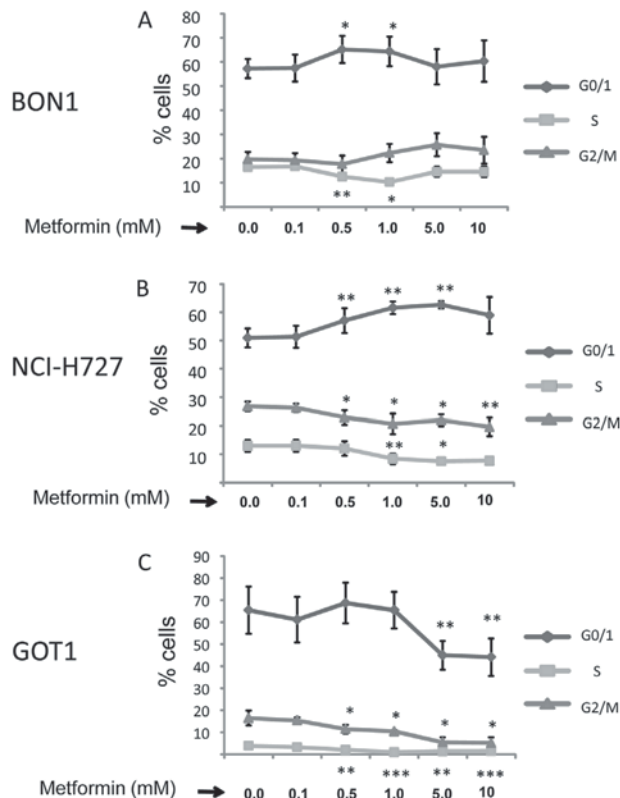
**Figure 5.** Effect of metformin on GSK3 / ERK1/2 signaling and apoptosis induction in neuroendocrine tumor cells. Human pancreatic BON1 (A), bronchopulmonary NCI-H727 (B) and midgut GOT1 (C) neuroendocrine tumor cells were treated with indicated concentrations of metformin for 24 and 48 hrs, respectively. Subsequently the expression of pGSK3, GSK3, pERK1/2, ERK1/2, EGFR and  $\beta$ -actin loading control was evaluated by Western blot analysis. A representative blot out of 3 independently performed experiments is shown. Human midgut neuroendocrine GOT1 cells cultured in complete medium were treated with the indicated concentrations of metformin (Figure 5D). After 72 hrs the proportion of cells in subG0/1 phase was examined by flow cytometry (left panel). Demonstrated are the mean values  $\pm$  SD of 2 independently performed experiments in duplicates (N=4); \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus untreated control. The expression of total / cleaved PARP and  $\beta$ -actin loading control was evaluated by Western blot analysis (24 and 48 hrs; right panel). A representative blot out of 3 independently performed experiments is shown.

responded with a dose-dependent decrease of cells in all three phases of the cell cycle (Figure 6C), likely due to strong induction of apoptosis (Figure 5D).

## DISCUSSION

Current systemic treatment options of neuroendocrine tumors include biotherapy with somatostatin analogues and interferon- $\alpha$ , molecular targeted therapy with everolimus and sunitinib, cytotoxic chemotherapy protocols, and peptide receptor-targeted

therapy.<sup>22,26,35</sup> Target-directed therapies such as the antiangiogenic multi-tyrosine kinase inhibitor sunitinib<sup>23</sup> and the mTOR inhibitor everolimus<sup>24,25</sup> have increased the therapeutic spectrum for a subset of progressive NET.<sup>22,26</sup> However, novel therapeutics are needed for sporadic NETs and particularly for NETs associated with hereditary syndromes such as MEN1 characterized by early onset and multiplicity of lesions.<sup>36,37</sup> Although the widely used antidiabetic drug metformin appears to exert potent anticancer effects in multiple types of cancer,<sup>29,30,34</sup> potential



**Figure 6.** Effect of metformin on cell cycle distribution of neuroendocrine tumor cells. Human pancreatic BON1 (A), bronchopulmonary NCI-H727 (B) and midgut GOT1 (C) neuroendocrine tumor cells were treated with the indicated concentrations of metformin for 72 hrs. Demonstrated are the mean values  $\pm$  SD of 3 independently performed experiments in duplicates (n=6); \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001 versus untreated control.

effects on NET have not been explored so far. As inhibition of the mTORC1 signaling by everolimus has already been proven a promising target for antitumoral therapy in NETs<sup>23-25,27</sup> and metformin also inhibits mTORC1 signaling,<sup>28-30</sup> potential effects of metformin on NETs were investigated.

In addition to indirectly-reached insulin-dependent effects of metformin (positive effects of lowered insulin levels on cancer), metformin appears to act through AMPK-dependent and AMPK-independent pathways.<sup>29,30,34</sup> Metformin leads to tumor suppressor liver kinase B1 (LKB1)-mediated activation of AMPK activity. This in turn suppresses mTORC1 signaling and protein synthesis through activation of tuberous sclerosis complex 2 (TSC2) or inhibition of

raptor.<sup>29,30</sup> In addition to AMPK-dependent reduction of mTORC1 signaling, metformin-mediated AMPK activation results in p53 phosphorylation and p53-mediated cell cycle arrest or apoptosis, depending on the p53 mutational status and cell type-dependent p53 signaling.<sup>29,30</sup>

AMPK-independent effects of metformin include Rag GTPase-mediated inhibition of mTORC1, reduced production of tumor necrosis factor alpha (TNF $\alpha$ ) and p53/p21-mediated inhibition of cyclin D1, and retinoblastoma-protein (pRb) and G1 cell cycle arrest.<sup>29,30</sup> The complexity of metformin signaling is also highlighted by the fact that, depending on the cancer cell type, inhibition of cell proliferation may be associated with G0/1, G2/M or even S phase arrest.<sup>34</sup>

In this study, the effect of metformin was examined on cell proliferation / viability in three human NET cell lines from pancreatic (BON1), bronchopulmonary (NCI-H727), and midgut (GOT1) origin. Treatment with increasing metformin concentrations (0.1 to 10 mM) dose-dependently suppressed cell proliferation / viability in all three cell lines. To further explore the mechanism of metformin action in NET, we examined the effect of metformin on AMPK phosphorylation. Pancreatic BON1 and particularly midgut GOT1 neuroendocrine tumor cells reacted with increased AMPK phosphorylation in response to metformin treatment, indicating that the observed antiproliferative effects were at least in part mediated through an AMPK-dependent mechanism. In contrast, bronchopulmonary NCI-H727 cells responded to metformin with decreased AMPK phosphorylation, suggesting involvement of AMPK-independent signaling.

Based on the central role of the PI3K/AKT/mTORC1 pathway for neuroendocrine tumor cell growth<sup>22,26</sup> and demonstrated (AMPK-dependent and -independent) suppression by metformin in cancer,<sup>29,30</sup> we examined the effect of metformin on neuroendocrine mTORC1 downstream targets. Treatment with increasing doses of metformin suppressed baseline phosphorylation of 4EBP1, P70S6K and S6 in all three neuroendocrine cell lines tested, suggesting that metformin-mediated inhibition of cell proliferation is mediated by inhibition of mTORC1 signaling. In NCI-H727 cells, lower metformin doses induced whereas higher doses suppressed AKT activity, while BON1 and GOT1 cells responded with a



dose-dependent increase in AKT phosphorylation at all time points tested. This argues in favor of cell type-dependent compensatory AKT activation in response to mTORC1 inhibition by metformin, similar to compensatory effects on AKT signaling as observed in response to everolimus.<sup>38,39</sup>

The complexity of metformin signaling in neuroendocrine cells of different origin is further highlighted by differences observed on ERK activity. In pancreatic BON1 and bronchopulmonary NCI-H727 cells, metformin potently induced ERK activity, while in midgut GOT1 cells it suppressed ERK phosphorylation. Failure of compensatory ERK activation in GOT1 cells could partially account for the more potent antiproliferative effect of metformin observed in these cells. It is likely that ERK (and AKT) activation in response to treatment with metformin reflects compensatory mechanisms of the tumor cell machinery responding to inhibition of mTORC1 signaling. This indicates that the effectiveness of combined treatments simultaneously blocking several signaling pathways may depend on the distinct neuroendocrine tumor signaling profile. This phenomenon appears to be cell type-specific, as treatment of PANC-1 or MiaPaCa-2 pancreatic cancer cells with metformin did not induce AKT and / or ERK activity (in contrast to rapamycin or active-site mTOR inhibitors).<sup>17</sup> It is not clear whether these compensatory mechanisms in response to mTOR inhibition are a sign of clinical resistance<sup>39,40</sup> or a sign of effectiveness of treatment.<sup>41</sup> In any case, simultaneous inhibition of mTOR and AKT (or ERK) signaling could potentiate the inhibitory effect on cell proliferation in GEP NET.<sup>39</sup>

Although metformin exerted varying effects on ERK / AKT activity depending on the neuroendocrine cell type tested, suppression of mTORC1 signaling was associated with induction of GSK3 phosphorylation in all three neuroendocrine cell lines. As GSK-3-mediated phosphorylation usually suppresses the activity of downstream targets involved in multiple intracellular signaling pathways, including cell proliferation and apoptosis,<sup>42-44</sup> ERK-<sup>45</sup> or AKT-dependent<sup>46</sup> GSK3 phosphorylation appears critical for metformin signaling in neuroendocrine tumor cells.

In pancreatic BON1 and bronchopulmonary NCI-H727 neuroendocrine tumor cells, metformin-mediated inhibition of cell proliferation was not associated

with induction of apoptosis, as metformin did not increase the number of cells in sub-G0/1 nor induce PARP cleavage, but rather with alterations in cell cycle distribution with increased G0/1 and decreased S phase entry. In contrast, the metformin-mediated antiproliferative effect in midgut GOT1 cells was, at least in part, associated with apoptosis induction, evidenced by increased sub-G0/1 events and PARP cleavage. Induction of apoptosis in GOT1 cells may be associated with the observed inhibition (rather than compensatory activation) of ERK signaling (similar to findings in pheochromocytoma cell lines<sup>47</sup>) and explain the more potent antiproliferative effect of metformin on this neuroendocrine tumor cell line.

Previously reported data from preclinical studies employing metformin tested relatively high metformin concentrations in vitro (1-40 mM (165-6600 mg/l);<sup>48-51</sup>) when compared to therapeutic plasma levels in human (2.8-15  $\mu$ M (0.465-2.5 mg/l);<sup>30</sup>). In acute metformin overdose in humans, survivors had a median peak metformin level of 42  $\mu$ g/ml vs 110  $\mu$ g/ml in non-survivors,<sup>52</sup> both concentrations ranging below 1 mM metformin. On the other hand, higher metformin concentrations may be necessary in vitro since cancer cells are cultured in complete medium containing extremely high amounts of growth factors and glucose. Metformin may accumulate in tissues particularly of the gastrointestinal system at much higher concentrations after oral or intravenous administration.<sup>53</sup> Further preclinical in vivo studies evaluating a wider spectrum of metformin dosages are needed to test the efficacy of metformin in neuroendocrine tumors, while considering dose-related side effects, such as development of lactic acidosis. The first preliminary clinical data on the role of metformin in recurrence-free survival in patients with neuroendocrine tumors have been reported recently.<sup>54</sup> In a retrospective analysis of 12 diabetic NET patients with metformin versus 24 non-diabetic NET patients without metformin, recurrence rate was lower by 8 versus 42% and recurrence-free survival was not attained compared to 86 months of the second group.<sup>54</sup>

This study demonstrates metformin-mediated inhibition of neuroendocrine tumor cell growth and signaling. Considering the good tolerability of the drug, the high rate of diabetes development of patients with NET under somatostatin analogue and

everolimus treatment, and the current lack of effective pharmacologic adjuvant therapies, further studies are needed to explore the potential use of metformin in patients harboring neuroendocrine tumors.

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### DISCLOSURE STATEMENT

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