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Compensatory activation of Akt in response to mTOR and Raf inhibitors – A rationale for dual-targeted therapy approaches in neuroendocrine tumor disease

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ABSTRACT

Several studies have established a link between aberrant PI(3)K–Akt–mTOR- and Ras–Raf–MEK–Erk1/2 signaling and neuroendocrine tumor disease. In this study, we comparatively investigate the antitumor potential of novel small-molecule inhibitors targeting mTOR (RAD001), mTOR/PI(3)K (NVP-BEZ235) and Raf (Raf265) on human NET cell lines of heterogeneous origin. All inhibitors induced potent antitumor effects which involved the induction of apoptosis and G0/G1 arrest. However, the dual mTOR/PI(3)K inhibitor NVP-BEZ235 was more efficient compared to the single mTOR inhibitor RAD001. Consistently, NVP-BEZ235 prevented the negative feedback activation of Akt as observed after treatment with RAD001. Raf265 inhibited Erk1/2 phosphorylation but strongly induced Akt phosphorylation and VEGF secretion, suggesting the existence of a compensatory feedback loop on PI3K-Akt signaling. Finally, combined treatment with RAD001 or NVP-BEZ235 and Raf265 was more efficient than single treatment with either kinase inhibitor. Together, our data provide a rationale for dual targeting of PI(3)K–Akt–mTOR- and Ras–Raf–MEK–Erk1/2 signaling in NET disease.

1. Introduction

Neuroendocrine tumors (NETs) of the gastroenteropancreatic (GEP) system are a rare and heterogeneous group of tumors. At the time of diagnosis, the majority of NETs have already metastasized, accounting for a rather low 5-year survival rate of less than 50% [1,2]. As currently available antiproliferative strategies against GEP-NETs (biotherapy, chemotherapy) have modest efficacy, novel therapeutic approaches are urgently needed.

The PI(3)K–Akt–mTOR- and the Ras–Raf–MEK-Erk1/2 pathway are crucial for the regulation of cell survival and proliferation. Growth factors initiate both signaling pathways by activating receptor tyrosine kinases (RTKs), which in turn leads to the activation of PI(3)K and its downstream targets Akt and mTOR on one hand and Ras and its downstream targets Raf, MEK and ERK1/2 on the other hand [3]. Akt, as well as Erk1/2 promote cell survival and proliferation by either directly or indirectly downregulating proapoptotic and cell cycle-inhibitory proteins such as Bim, Bad, p27 and p21. Conversely, Akt and Erk1/2 upregulate antiapoptotic and cell cycle promoting proteins such as Bcl-2, Bcl-XL, c-Myc and cyclin D1 [4]. One major target of Akt is the mTORC1, which is composed of mTOR, regulatory-associated protein of mTOR (raptor) and mLST8. Two well-characterized mTORC1 substrates are eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4EBP1) and p70 ribosomal S6 kinase (p70S6K), both regulating transcription and translation initiation of critical growth genes. Moreover, p70S6K is part of a powerful

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negative feedback loop on PI(3)K–Akt signaling [5]. The second mTOR-containing complex, mTORC2, consists of mTOR, rapamycin-insensitive companion of mTOR (rictor), Sin1, mLST8 and protein associated with Rictor (protor). It is less understood than mTORC1 but recent work indicates that it is part of the PI(3)K–Akt pathway as it mediates Akt phosphorylation on Ser473 which is required for full Akt activity [6,7].

There is accumulating evidence that the PI(3)K-Akt-mTOR- and the Ras–Raf–MEK–ERK1/2 pathway closely cooperate in the transduction of survival signals. For instance, Ras and PI(3)K can directly activate each other and Akt has been found to inhibit Raf [8]. In addition, a recent study by Carracedo et al. revealed a p70S6K-mediated negative feedback loop on Raf-MEK-Erk signaling [9].

The PI(3)K–Akt–mTOR- and the Ras–Raf–MEK–ERK1/2 pathways are among the major signaling networks that have been implicated in human cancer including NETs. Indeed, a recent study found that 76% of all examined NET samples were positive for activated Akt and 96% were positive for activated ERK1/2 [10]. Molecular analysis of NETs suggests that in addition to mutations in certain tumor suppressor genes (e.g. PTEN, B-Raf), autocrine growth factor loops contribute to hyperactive PI(3)K–Akt–mTOR- and Ras–Raf–MEK–ERK1/2 signaling. For instance, abnormal high or constitutive expression of IGF-I and IGF-IR-TK has been detected in the majority of NETs and considerably contributes to neuroendocrine secretion and tumor cell growth [11,12]. Not surprisingly, those insights have prompted several approaches of specifically targeting tyrosine and serine/threonine kinases along the P(3)K–Akt–mTOR- and Ras–Raf–MEK–ERK1/2 pathway. Among the huge number of selective small-molecule inhibitors that have been recently introduced for cancer therapy, several have already been tested in NETs. For instance, the mTOR inhibitor RAD001 and the Raf inhibitor sorafenib have both demonstrated potent antitumor activity *in vitro* and have recently been evaluated in patients with advanced NET disease [13–17]. Out of 60 patients receiving RAD001 orally 5 or 10 mg daily and depot octreotide intramusculary every 28 days, partial response (PR) or stable disease (SD) were observed in 22% and 70% of patients, respectively. In contrast, tumor response to sorafenib was modest with PR and SD rates of 10% and 50%, respectively.

Here, we comparatively test the antitumor potential of novel small-molecule-inhibitors specifically targeting mTOR (RAD001), mTOR/PI(3)K (NVP-BEZ235) and Raf (Raf265) in three NET cell lines of pancreatic, midgut and bronchial origin. Our results suggest the existence of a novel compensatory feedback mechanism between PI(3)K–Akt–mTOR- and Ras–Raf–MEK–ERK1/2 survival signaling and provide a rationale for dual targeting of these pathways in NET disease.

2. Materials and methods

2.1. Reagents

RAD001, NVP-BEZ235, RAF265 and NVP-AEW541 were kindly provided from Novartis Pharma (Basel, Switzerland).

2.2. Cell culture

Human pancreatic neuroendocrine BON1 tumor cells were kindly provided by R. Göke (Marburg, Germany) and cultured in DMEM/F12 (1:1) medium (Gibco/Invitrogen, Karlsruhe, Germany). Human midgut carcinoid GOT1 cells were kindly provided by Ola Nilsson (Göteborg, Sweden). Human bronchopulmonary neuroendocrine NCI-H727 tumor cells were purchased by ATCC (Manassas, VA, USA). GOT1 and NCI-H727 cells were cultured in RPMI medium (PAA, Pasching, Austria). All media were supplemented with 10% FCS (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Gibco) and 0.4% amphotericin B (Biochrom). GOT1 culture medium was additionally supplemented with 0.135 IU/ml insulin and 5 mg/ml apo-transferrin. All cells were cultured at 37 °C in a 5% CO_2 atmosphere.

2.3. Assessment of cell viability

BON1, GOT1 and NCI-H727 cells were seeded into 96-well plates at densities of 3000, 50,000 and 4000 cells per well and grown for 24 h. Next, the cells were incubated with various concentrations of NVP-BEZ235, RAD001, Raf265 or NVP-AEW541 in medium containing 10% FCS. Metabolic activity was measured with Cell Titer 96 aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) after 24 h and 72 h of incubation according to the manufacturer's instructions. Following 3 h of incubation with Cell Titer 96 solution, absorbance at 492 nm was determined using an ELISA plate reader.

In order to visualize the effects on cell numbers, cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.05% crystal violet in distilled water for 30 min. Subsequently, cells were washed with tap water twice before the plates were photographed.

2.4. Hoechst staining

For morphologic assessment of chromatin condensation and DNA fragmentation, cells were fixed with 4% paraformaldehyde for 20 min followed by washing with PBS. The fixed cells were stained with 250 μg/ml Hoechst 33258 (Sigma, St. Louis, MO, USA) in PBS for 1 h and subsequently examined by fluorescence microscopy.

2.5. Protein extraction and Western blotting

Protein extraction and Western blotting were done as previously described in detail [18]. Primary antibodies used were pAkt (Ser473), Akt, pMEK, MEK, pERK1/2, ERK1/2, pp70S6 K, p70S6 K, Bcl-xL, Cyclin D1, Cyclin D3, p27kip1 (Cell Signaling, Danvers, MA, USA); Bcl-2, p21 Waf1/Cip1 (BD, Franklin Lakes, NJ, USA) and β-actin (Abcam, Cambridge, UK).

2.6. Quantification of DNA fragmentation and cell cycle analysis

The rate of apoptotic cell death was quantified by determining DNA fragmentation according to Nicoletti et al. [19]. Briefly, cells were incubated for 24 h in a hypotonic