The oral multitarget tumour growth inhibitor, ZK 304709, inhibits growth of pancreatic neuroendocrine tumours in an orthotopic mouse model

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ABSTRACT

Background and aims: Current systemic therapies for neuroendocrine tumours (NETs) do not provide sufficient control of tumour growth. However, efficient evaluation of novel drugs is hindered by the lack of a suitable preclinical animal model. Here an orthotopic mouse model of pancreatic NET is established and used to study the action of ZK 304709, a first in class, oral multitarget tumour growth inhibitor. ZK 304709 is an inhibitor of cyclin-dependent kinases (Cdk) 1, 2, 4, 7 and 9, vascular endothelial growth factor receptor-type kinases (VEGF-RTKs) 1–3 and platelet-derived growth factor receptor-type kinase β (PDGF-RTKβ).

Methods: BON and GEP-1 human NET cells were used to study proliferation, survival and cell cycle distribution in vitro. For induction of orthotopic NETs, BON cells were injected into the pancreas of NMRInu/nu mice. Primary tumour growth and metastatic spread were recorded after 9 weeks, and apoptosis, microvessel density and lymphatic vessel density were determined.

Results: ZK 304709 dose-dependently suppressed proliferation and colony formation of NET cells. Direct effects on NET cells were consistent with Cdk inhibition and involved G0 cell cycle arrest and apoptosis induction, which was associated with reduced expression of MCL1 (myeloid cell leukaemia sequence 1), survivin and hypoxia-inducible factor 1α (HIF1α). Apoptosis similarly occurred in vivo in ZK 304709-treated orthotopic BON tumours, resulting in a 80% reduction of primary tumour growth. In contrast, treatment with laneotide or 5-fluorouracil and streptozotocin failed to inhibit tumour growth. ZK 304709 also reduced tumour microvessel density, implicating antiangiogenic mechanisms.

Conclusion: BON orthotopic tumours provide an informative model for preclinical drug evaluation in NETs. In this model, ZK 304709 achieved efficacious tumour growth control via induction of apoptosis and inhibition of tumour-induced angiogenesis.

Palliative management of gastroenteropancreatic neuroendocrine tumours (GEP-NETs) currently relies on biotherapy with somatostatin analogues and interferon α, on chemotherapy and on chemoembolisation of liver metastases.1 Given the low response rates and/or considerable side effects of these modalities, improved treatment options are needed.

Cyclin-dependent kinases (Cdk) have attracted considerable interest as drug targets in oncology.3 Cdk comprise a family of serine/threonine kinases with two major functions: they serve either as core components of the cell cycle machinery, where they promote and control transit between distinct cell cycle phases, or as regulators of RNA polymerase II activity, which participates in transcription control. Although perturbations of individual Cdks result in cell cycle arrest of tumour cells,2 recent studies indicated an unforeseen redundancy of Cdk–cyclin interactions.2 Moreover, apoptosis following pharmacological Cdk inhibition probably resulted from inhibition of transcriptional Cdks rather than cell cycle perturbation.2 In view of these findings, the paradigm of the ideal therapeutically active Cdk inhibitor has shifted from a highly selective inhibitor of an individual Cdk towards a compound that targets multiple members of the Cdk family with different functions.3

ZK 304709 is an oral small molecule kinase inhibitor of Cdks 1, 2, 4, 7 and 9 at nanomolar concentrations. In addition to these ubiquitous targets, ZK 304709 blocks the activity of vascular endothelial growth factor receptor-type kinases (VEGF-RTKs) 1–3 and platelet-derived growth factor receptor-type kinase β (PDGF-RTKβ) expressed on endothelial cells, thereby adding an antiangiogenic and possibly antilymphangiogenic action profile in vivo. Given the molecular heterogeneity11 and abundant vascularisation12 that represent well-established features of NETs, the multitargeted profile of ZK 304709 seems ideally suited for antitumour action in these malignancies.

Currently the lack of a reliable and representative animal model of GEP-NET disease limits an efficient preclinical evaluation of experimental therapeutics. Here, we established an orthotopic in vivo model of pancreatic neuroendocrine cancer and used this model to evaluate the therapeutic effects of ZK 304709.

MATERIALS AND METHODS

Antibodies were from the following sources: poly(ADP) ribose polymerase (PARP) from Oncogene Research Products (Bad Soden, Germany); pRB, cyclin E and CD84 from BD Pharmingen (Heidelberg, Germany); cdc2, cdk2 and human pancytokeratin (C11) from Santa Cruz Corporation (Santa Cruz, California, USA); survivin, hypoxia-inducible factor 1α (HIF1α) from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany); cyclin A, MPM2 (mitotic protein monoclonal 2) and myeloid cell leukaemia...
sequence 1 (MCL1) from Upstate Biotechnology (Charlottesville, Virginia, USA); synaptophysin and chromogranin A (CgA) from DAKO (Hamburg, Germany) and lymphatic vessel endothelial hyaluronan receptor (LYVE-1) from ReliaTech (Braunschweig, Germany). Secondary antibodies and anti-human Ki67 were from Dianova (Hamburg, Germany). Nocodazole, streptozotocin (STZ), fluorouracil (5-FU) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (Deisenhofen, Germany). Rompun was from Bayer GmbH (Leverkusen, Germany), Ketavet from Pharmacia GmbH (Erlangen, Germany) and lanreotide (SOMATULINE Autogel) was from Ipsen Pharma GmbH (Ettlingen, Germany).

**Cell culture**

BON cells were a generous gift from C M Townsend (Galveston, Texas, USA). QGP-1 cells were from the Health Science Research Resources Bank (Osaka, Japan). Both cell lines were derived from metastasised pancreatic NETs, but differ with respect to functionality and molecular alterations. Of note, QGP-1 cells are functionally deficient for the tumour suppressor pRb and BON cells for p16^ink4a_.14 15 16 Cells were cultured as described.16

**Growth assays**

Cells were plated in 96-well dishes at 2500 cells/well. At the indicated times, viable cells were counted in a haemocytometer. Clonal growth of NET cells was examined as described.16

**Flow cytometry and immunofluorescence**

For cell cycle analyses, adherent and detached cells were collected and DNA content was determined on a FACSCalibur utilising “Cellquest” software (Becton Dickensen, Heidelberg, Germany) as described. For MPM2 staining, cells were incubated with 1 μg/ml MPM2 antibody prior to propidium iodide staining. To assess nuclear morphology, cells were stained with 1 μg/ml DAPI after methanol–acetone fixation and examined using a Zeiss Axioplan.

**Immunoblotting**

A total of 5×10^6 cells were lysed in 1 ml of RIPA buffer supplemented with protease inhibitors and processed as previously described.16 For immunoblotting of PARP, 10^6 cells were lysed in 250 μl of urea buffer.17 Nuclear extracts were prepared using a non-ionic detergent method.18

**Tumour implantation**

Female NMRInu/nu mice (21–25 g) were from Bomholtgard (Ry, Denmark). Animal care followed institutional guidelines and experiments were approved by local animal research authorities. Mice were anaesthetised by intraperitoneal administration of Ketavet (100 mg/kg) and Rompun (10 mg/kg). For tumour induction, the pancreas was exposed and 1×10^6 BON cells were injected into the head of the pancreas. After 9 weeks, mice were killed, blood samples were collected and primary tumours were removed and weighed. Tumour volume was calculated using the formula length × width × depth × π/6. Enlarged lymph nodes and macroscopically evident metastases were collected for histology. Also, liver tissue and liver hilus lymph nodes were routinely obtained.

**Histochemical analyses**

Cryostat sections (4 μm) of murine tissues were fixed in 4% paraformaldehyde, pH 7. Immunoperoxidase staining was performed with an avidin–biotin complex method (Vestacastain Elite ABC kit, Vector Laboratories, Wertheim-Bettingen, Germany) using 3-amino-9-ethylcarbazole (AEC, DAKO, Hamburg, Germany) substrate. Antibody dilutions were: CD31, 1:100; pancytokeratin, 1:50; LYVE-1, 1:200; CgA, 1:300; and synaptophysin, 1:100. Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay using the TumorTACS in situ Apoptosis Detection Kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). Primary antibodies or terminal deoxynucleotidyl transferase, respectively, were omitted in negative controls. Quantitative immunohistochemical analysis was carried out using Axiovision 4.2 software (Zeiss, Jena, Germany).

**Figure 1** ZK 304709 inhibits the proliferation of BON (squares) and QGP-1 (circles) in neuroendocrine cells. Subconfluent cells were treated with 500 nM ZK 304709 or 100 nM octreotide (A), or with the indicated concentrations of ZK 304709 (B) for 72 h and numbers of viable cells were determined. (A) ZK 304709 reduced cell numbers to 10% and 2% of untreated controls, which corresponded to 664 and 267 cells/well and was below the initial plating number of 2500 cells/well. Shown are means and 95% CIs (*p<0.05). (B) Growth inhibition by ZK 304709 occurred at nanomolar concentrations. Shown are means (SEM). (C) A single cell agar suspension was incubated with the indicated doses of ZK 304709 for 10 days and colony formation was evaluated. Colonies were reduced to 4.7% (0.7%) of control in BON and 3% (1%) in QGP-1 cells at the maximal concentration of 500 nM ZK 304709. Data represent means and 95% CIs. Data in A–C are from three separate experiments, with each determination conducted in triplicate.

**Pancreatic disease**

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Determination of microvessel density and lymphatic microvessel density
For quantitation of microvessel density (MVD) and lymphatic vessel density, respectively, the average number of CD34- and LYVE-1-positive vessels in a 0.6 mm$^2$ measurement area was determined from three regions of maximal vascular density (vascular hotspots).\(^{19}\)

Determination of chromogranin A serum concentration
Serum CgA levels were measured using a CgA ELISA Kit (Dako Cytomation GmbH) as recommended by the manufacturer.

Statistical analysis
Data analysis was performed by analysis of variance (ANOVA) utilising the Newman–Keuls test, Mann–Whitney or Fisher exact test (graph pad software, San Diego, California, USA). Differences were considered significant at p<0.05.

RESULTS
ZK 304709 inhibits anchorage-dependent and anchorage-independent growth of human NET cells
The kinase inhibition profile of ZK 304709 has been extensively characterised in cell-free systems, but potential mechanisms of antitumour action still await confirmation at the cellular level. Treatment of human pancreatic NET cell lines with 500 nM ZK 304709 for 72 h severely reduced cell numbers, whereas 100 nM octreotide had only minor effects (fig 1A). Growth inhibition was concentration dependent, with a calculated EC$_{50}$ (median effective concentration) of 129 nM for BON and 79 nM for QGP-1 cells (fig 1B). At 500 nM, ZK 304709 reduced cell
numbers even below initial numbers, suggesting that cell death occurred.

We next evaluated colony formation in agar suspension (fig 1C). ZK 304709 suppressed colony formation by approximately 95%, with EC50 values comparable with those in the proliferation studies.

Thus, ZK 304709 elicited profound growth inhibitory effects at concentrations compatible with inhibition of Cdk complexes in biochemical kinase assays.

**ZK 304709 treatment of NET cells results in G2/M arrest and apoptosis**

In cell cycle analyses, the growth inhibition of ZK 304709-treated cells was reflected by a time-dependent increase in the G2/M phase population and concomitant reduction of cells in the S-phase (fig 2A). An analysis of MPM2 mitotic epitopes allowed the increase to be allocated more precisely to the G2 phase, consistent with the increased size of DAPI-stained nuclei (fig 2B). Prolonged treatment increased the pre-G1 fraction, indicative of apoptosis induction (fig 2C). Apoptosis was confirmed at the biochemical level by detection of caspase-cleaved PARP (fig 2D). Comparable changes were observed in QGP-1 cells, though apoptosis started as early as 16 h of treatment (fig 3). PTK-ZK, a compound with a similar spectrum of inhibitory activities against VEGF family RTKs, had no effect on cell cycle distribution (figs 2 and 3), suggesting that ZK 304709's effects on tumour cells resulted from Cdk inhibition.

Although QGP-1 and BON cells differ with respect to pRb expression, ZK 304709-induced cell cycle redistribution was similar in both lines. As this observation argued against a decisive contribution of cyclin D–Cdk4/6 complexes, further analysis focused on Cdk2 and Cdc2 complexes. Immunoblotting of NET cell lines revealed an increased cyclin E complement (1.4 (0.02) times control in BON and 1.94 (0.3) times control in QGP-1 cells at 24 h, p<0.001 and p<0.05, respectively), while levels of neither Cdns nor Cdk inhibitors p21 or p27 were altered (fig 4A and data not shown).

Taken together, DNA histograms and regulatory changes of cyclins supported an inhibition of Cdc2 and Cdk2 complexes, as was expected from the action profile of ZK 304709 in cell-free assays.

**ZK 304709-induced apoptosis is associated with the depletion of endogenous apoptosis inhibitors**

Apoptosis induction by Cdk inhibitors has been linked to inhibition of transcriptional Cdns with subsequent depletion of short-lived apoptosis inhibitors such as MCL1. Immunoblot analysis of MCL1 expression in ZK 304709-treated NET cells indeed illustrated a rapid decrease (45% (6.1%) of control in BON and 31% (9.5%) of control in QGP-1 cells, p<0.05 at 6 h, fig 4B, left), which persisted throughout 48 h (fig 4B, right).

Survivin represents a second apoptosis-protective target of Cdk inhibitors. Again, immunoblot analyses revealed a reduction of cellular survivin content in both NET lines, with a maximal inhibition to 33% (7.9%) of the control observed at 24 h in QGP-1 cells and to 56% (9%) at 48 h in BON cells (p<0.05, data not shown). Because nuclear, but not cytoplasmic survivin expression was linked to prognosis of NETs in immunohistochemical studies, we separately analysed both compartments. Upon ZK 304709 treatment, the survivin complement of the nuclear fraction decreased in both cell lines (fig 4C).

More recently, the transcription factor HIF1α was identified as a potential target of Cdk inhibitors. HIF1α mediates adaptive responses to hypoxia and to the tumour cell’s increased energy demand, and thereby contributes to apoptosis protection. HIF1α protein was easily detected in nuclear extracts of NET cells under normoxia and accumulated following chemical induction with CoCl2 (fig 4D). Upon treatment with ZK 304709 for 16 h, basal levels of HIF1α declined to 36% (8%) of control in QGP-1 cells and to 56% (9%) in BON cells (n = 3–4, p<0.05). In contrast, HIF1α stabilisation due to hypoxia or CoCl2 remained unaffected (data not shown).

Figure 3  ZK 304709 treatment of QGP-1 cells results in G2 cell cycle arrest and apoptosis induction. (A) Randomly cycling QGP-1 cells were treated with 500 nM ZK 304709 or vehicle for the indicated times, and cell cycle analyses were performed. Shown are DNA histograms representative of at least three independent experiments. (B) Cells were treated with 500 nM ZK 304709 or vehicle for 48 h and apoptotic poly(ADP) ribose polymerase (PARP) cleavage was ascertained by immunoblot analyses documenting the presence of the 85 kDa PARP cleavage product in ZK 304709-treated cells.
Overall, ZK 304709-mediated depletion of multiple apoptosis inhibitors probably facilitated apoptosis induction by lowering the apoptotic threshold.

**BON orthotopic tumours in mice represent a useful model system to study NET biology**

Based on these encouraging results, we addressed the anti-tumour action of ZK 304709 in vivo. In contrast to the in vitro situation, we anticipated a relevant contribution of the ability of ZK 304709 to inhibit proangiogenic receptors. Accordingly, an in vivo model, which permitted examination of primary tumour growth, tumour neoangiogenesis and metastatic spread, seemed mandatory.

We therefore adapted the procedure for orthotopic xenografting of exocrine pancreatic cancer cells and implanted human pancreatic BON NET cells, known to form subcutaneous tumours in nude mice into the pancreas of NMRI nu/nu mice.

Following implantation, we monitored tumour growth at 5, 7 and 9 weeks (fig 5A,B). Tumours developed in >90% of animals. By 9 weeks, they had reached >300 mg average weight, and individual tumours presented with signs of locally invasive growth into adjacent duodenal or gastric tissues and Ki67 fractions ranging from 1% to 7% of cells (median 2.4). Orthotopic BON tumours retained markers of neuroendocrine differentiation such as CgA and synaptophysin (fig 5C). Furthermore, antibodies against the endothelial marker CD34 or against LYVE-1, a specific epitope of lymphendothelial cells, revealed abundant vasculature, indicating that BON tumours reflected this established NET feature (fig 5C, lower panel, left).
Occasional tumour cells were noted in lymphatic vessels, suggesting infiltration of lymphatics (fig 5C, lower panel, right). Finally, CgA serum concentrations at autopsy correlated with tumour mass, thus providing a potential biomarker for non-invasive monitoring of BON tumours (fig 5D).

**BON orthotopic NETs model metastatic disease**

Besides the primary tumour, suspect intra-abdominal lesions were recorded, suggesting metastasis occurred (fig 6). Detection of human cytokeratin (fig 6A, lower right) and neuroendocrine differentiation markers in these lesions (fig 6A, lower right) corroborated metastasis formation. Prevalent sites of metastasis involved liver and abdominal lymph nodes (fig 6A,B), although additional locations (eg, peritoneum) were noted in individual cases.

**ZK 304709 inhibits tumour growth in BON orthotopic NETs**

Having established BON orthotopic tumours as an appropriate in vivo model, we evaluated the antitumour potential of ZK 304709. Tumours were allowed to grow for 2 weeks, before animals were randomly assigned to either the treatment group (100 mg/kg/day ZK 304709 orally), a control group (vehicle) or to a section group, which was sacrificed at the start of the treatment in order to determine average tumour burden. Treatment continued for 7 weeks, until mice were sacrificed. By that time, tumour weight in the treatment group was significantly reduced (fig 7A), which was reflected by decreased tumour volumes (113.5 (25.92) mm$^3$ vs 611.7 (96.33) mm$^3$ in controls, $p<0.01$) and a reduction in the plasma CgA concentration (fig 7B). Furthermore, fewer ZK 304709-treated animals presented macrometastases (fig 7C), although the difference did not reach statistical significance due to the small number of animals that could be evaluated.
ZK 304709 reduces microvessel density of BON orthotopic NETs

In order to assess whether ZK 304709 affected tumour growth indirectly by targeting the endothelial compartment, MVD and lymphatic vessel density were determined via immunohistochemical detection of CD34 and LYVE-1, respectively (fig 8B). A significant reduction of MVD had occurred in ZK 304709-treated animals, indicating an inhibition of tumourangiogenesis (fig 8B upper panel). A similar trend was noted for lymphatic vessel density, though the reduction failed to reach statistical significance (fig 8B, lower panel).

DISCUSSION

Despite improved clinical management of GEP-NETs, treatment modalities do not yet provide satisfactory control of tumour growth. Two obstacles impede progress: first, low proliferation rates account for poor responses to conventional chemotherapies and a narrow therapeutic window. Secondly, the molecular diversity of NETs has until recently precluded the identification of disease-specific molecular alterations, which could then translate into the development of a specific targeted drug.

Between these limitations, a number of targeted therapies, which interfere with specific components of commonly altered proangiogenic, mitogenic and antiapoptotic signalling pathways, are currently being tested as single agents or in combination (http://www.cancer.gov/clinicaltrials).

We pursued a different approach, using the multitargeted kinase inhibitor ZK 304709, which acts on multiple Cdkks and on endothelial RTKs. ZK 304709 has shown superior antitumour efficacy in diverse xenograft models including breast, colonic, pancreatic and kidney cancer when compared with standard chemotherapy, and has recently undergone phase I clinical trials. In NET cells, ZK 304709 inhibited growth at nanomolar concentrations in vitro, via mechanisms that involved G2 cell cycle inhibition and induction of apoptosis. These effects translated into tumour cell apoptosis and inhibition of tumourangiogenesis in an orthotopic mouse model of NET disease, resulting in a profound reduction of tumour burden.

Unlike earlier concepts on the use of Cdk inhibitors, which aimed to re-establish pRb-like checkpoint control via inhibition of G1 kinases, recent insights into cell cycle control of tumour cells advocate a combination approach targeting both Cdk2 and Cdc2, leading to cell cycle arrest in the G2 phase. In fact, such a pattern of G2 accumulation occurred in ZK 304709-treated NET cells at drug concentrations which inhibited both Cdk2 and Cdc2 in biochemical kinase inhibition studies. Cell cycle effects did not differ between pRb-competent BON cells, and pRb-deficient GQG-1 cells, indicating that Cdk4/Cdk2-mediated, pRb-dependent mechanisms were dispensable for the cell cycle inhibitory activity of ZK 304709.

Apart from the G2 cell cycle arrest, ZK 304709 induced apoptosis, and thus converted a reversible inhibition of Cdk activity into irreversible tumour cell death. Apoptosis following Cdk inhibition potentially results from deregulated, increased E2F activity, or from inhibition of the transcriptional Cdkks and/or 9. Complexed to cyclin H and cyclin T, respectively, Cdkks 7 and 9 activate RNA polymerase II. Therefore, their inhibition leads to depletion of short-lived mRNAs including apoptosis inhibitors, as was shown for the antiapoptotic bcl-2 family member MCL1 following pharmacological ablation of Cdk9 activity by either flavopiridol or roscovitine. In NET cells we similarly noted an early and persistent reduction of MCL1 following ZK 304709 treatment, suggesting that the drug affected the balance of proapoptotic and antiapoptotic bcl-2 family members and thereby lowered the apoptotic threshold.

ZK 304709 induces tumour cell apoptosis in BON orthotopic NETs

Given the convincing antitumour action of ZK 304709, we aimed to outline mechanisms that acted in vivo. A relevant contribution of apoptosis was evident from in situ TUNEL staining, which documented a sixfold increased apoptotic index in tumours from the treatment group (fig 8A, left).

We then sought to compare the efficacy of ZK 304709 with that of established treatment modalities. Thus, nude mice bearing BON tumours received either ZK 304709 as described above, or lanreotide or a combination chemotherapy of 5-FU and STZ. Drug dosage and application schedule followed the protocol used for palliative treatment of NETs, with the notable exception of STZ, which causes severe β-cell toxicity in mice and was accordingly reduced in order to avoid diabetes induction. Both lanreotide and 5-FU/STZ treatment were well tolerated, as judged based on comparable body weights (not shown), but did not delay tumour growth, whereas ZK 304709 had again significantly reduced tumour burden (fig 7D).

Figure 6  Illustration and summary of the characteristic pathological features of BON orthotopic neuroendocrine tumours. (A) Histological and macroscopic evaluation of neuroendocrine metastases from BON orthotopic tumours. Immunohistochemistry ascertains metastasis based on detection of human cytokerin and synaptophysin. Scale bars indicate 200 and 2 mm in H&E stains of liver metastasis and lymph node metastasis, and 200 and 500 μm in immunohistochemistry for synaptophysin and cytokerin. (B) Summary of pathological features from BON orthotopic tumours in nude mice. Data are presented as a percentage of animals. Evaluation is based on macroscopic pathology with respect to liver metastasis and on the presence of cytokerin in histopathological examination for determination of lymph node metastasis, n = 18.
While the biological significance of MCL1 regulation in NETs is unclear, nuclear expression of the apoptosis inhibitor survivin has been connected to poor differentiation and metastasis of NETs. We found survivin expressed in both NET cell lines in the nuclear and the cytosolic compartment and noted a reduction upon ZK 304709 exposure, an effect which may have contributed to the altered apoptotic threshold observed. Reduced expression of survivin by Cdk inhibitors was previously proposed to result from transcriptional inhibition and/or increased proteasomal degradation, possibly a consequence of cdc2 inhibition.

We also observed an inhibition of nuclear HIF1α expression in ZK 304709-treated cells under normoxic conditions, when HIF1α’s presence may depend on oncogene-driven transcriptional control rather than protein stabilisation. Given that flavopiridol downregulated HIF1α via proteasome-independent mechanisms in human glioma cells, HIF1α may represent a common target of Cdk inhibitors, though the exact mechanism and the functional relevance of HIF1α reduction in NETs remain to be determined.

Considering that ZK 304709 affected multiple and diverse signalling pathways, its almost uniform in vitro action in BON and QGP-1 cells is notable, since both cell lines differ in their molecular profile with respect to the tumour suppressors p16 and pRb to bcl-2 and to transforming growth factor β effectors. Their comparable responsiveness to ZK 304709 thus supports the concept of targeting the core cell cycle and transcription machinery in molecularly heterogeneous malignancies such as NETs.

Based on our in vitro data, apoptosis induction represented an integral aspect of the growth-inhibitory action of ZK 304709 and converted a reversible inhibition of Cdk activity into irreversible tumour cell death. Even more importantly, tumour cell apoptosis represented a relevant antitumour mechanism in vivo at drug concentrations achieved by oral application of ZK 304709. In contrast, we found no evidence for apoptotic cell death in the liver or residual non-transformed pancreatic tissue, consistent with the proapoptotic mechanisms operating predominantly in transformed cells.

In addition to apoptosis, we noted a reduction of MVD in treated animals. As BON cells express and secrete high levels of VEGF-A, and ZK 304709 can block signalling via VEGFR1 and 2, this observation conceivably reflected a disruption of...
proangiogenic VEGF-A signalling. Lymphangiogenic signalling via VEGFR3 should similarly be compromised by ZK 304709 treatment. Major VEGFR3 ligands are VEGF-C and VEGF-D, which are both prominently expressed in non-transformed neuroendocrine cells, suggesting a physiological role in neuroendocrine differentiation. In a large series of human pancreatic NET specimens, overexpression of VEGF-C, but not VEGF-D expression, was specifically associated with lymphangiogenesis and lymph vessel invasion, which occurred more frequently in angioinvasive/metastatic tumours. Thus, reduction of lymphangiogenesis possibly constitutes an added clinical benefit of pharmacological inactivation of VEGFR3 by ZK 304709. In the current study we noted a trend toward lymphatic vessel density reduction, raising the possibility that a reduction of lymph vascularisation contributed to the antitumour action of ZK 304709 in some mice.

While the concept of an antiangiogenic treatment for NETs is accepted as particularly promising, clinical results have remained ambiguous. For instance, endostatin failed in a phase II trial in pancreatic NETs, although the substance was highly efficacious in the rip-tag model of multistep islet cell carcinogenesis. An intriguing challenge is, furthermore, provided by the inverse correlation of MVD and disease progression reported from clinical specimens of pancreatic NETs. Thus, additional in vivo models of NETs may prove helpful in both preclinical drug testing and dissecting the biology of NET vascularisation.

The current orthotopic model uses a representative human NET cell line in a microenvironment which offers an organotypical interaction between the tumour cells, the stroma, the vascular compartment and the drug. Thus, it provides a hitherto unique opportunity to study context-driven aspects of human NET biology and drug response, such as metastasis, which cannot be addressed in the subcutaneous setting. Neither lanreotide biotherapy nor 5-FU/STZ chemotherapy were able to control tumour growth in this orthotopic model, reflecting the unresolved medical needs of advanced metastatic pancreatic NETs. Our first preclinical evaluation of a novel treatment modality in this model suggests ZK 304709 as an attractive treatment alternative for human NETs.

Figure 8  Mechanisms of ZK 304709 antitumour action in vivo. (A) Determination of ZK 304709 effects on tumour cell apoptosis. Apoptosis fractions were measured by in situ TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) on tissue sections of the primary tumour. Shown are representative TUNEL stainings from a ZK 304709-treated tumour and a vehicle-treated control tumour. Scale bar = 100 μm. A quantitative analysis of the results was obtained by computer-aided image analysis. Apoptotic cells were counted in 20 randomly selected measurement areas (680×510 μm, magnification ×20) of each specimen and expressed as a percentage of total tumour cells. The graph on the right summarises the quantitative analysis of the histochemical results. (B) Effects of ZK 304709 on lymphangiogenesis. Representative immunohistochemical analyses of microvessel density (MVD; CD34, left) and lymphatic vessel density (LVD; lymphatic vessel endothelial hyaluronan receptor (LYVE-1), right) in BON tumours from control- and ZK 304709-treated animals. Shown are areas of high vessel density (hot spots), which were used for quantification of MVD and LVD. Average numbers of CD34- and LYVE-1-positive vessels were counted from three vascular hotspots. Results for MVD and LVD are given per mm² and were summarised in the graph, which shows the median, upper and lower quartile and upper and lower extreme for each group. t Test was used to calculate p values between groups. Scale bars correspond to 100 μm in (A) and 300 μm in (B).
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