Treatment of biliary tract cancer with NVP-AEW541: Mechanisms of action and resistance

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NVP-AEW541 was associated with dephosphorylation of IGF-1R and AKT. In contrast, phosphorylation of p42/p44 and Stat3 and expression of Bcl-xL were inconsistently downregulated. In addition, treated cells showed cell cycle arrest at the G1/S-checkpoint and an increase in sub-G1 peak. Moreover, IGF-1R and its ligands IGF-1 and IGF-2 were co-expressed in RT-PCR, suggesting an autocrine loop of tumor cell activation. Combined with gemcitabine, NVP-AEW541 exerted synergistic effects, particularly at low concentrations, while effects of combination with 5-FU or BI 2536 were only additive.

CONCLUSION: Our findings suggest that NVP-AEW541 is active against BTC in vitro and potentiates the efficacy of gemcitabine.

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Key words: Tyrosine kinase inhibitor; Cholangiocarcinoma; Gemcitabine; NVP-AEW541

INTRODUCTION

Insulin-like growth factor-1 receptor (IGF-1R) is a tyrosine kinase receptor with a 70% homology to the insulin receptor[1]. When activated by its ligands IGF-1, IGF-2 or insulin at supraphysiological concentrations, the IGF-1R transmits a signal to its two major substrates, insulin receptor substrate-1 (IRS-1) and Shc. The signal is subsequently transduced via the common signal transduction...
pathway, through ras, raf and p42/44 downstream of She and AKT downstream of IRS-1, all the way to the nucleus\[2,3\].

The IGF-1R system has emerged as an interesting target for cancer therapy, as it represents an important promotor of tumor transformation and survival of malignant cells, but is only partially involved in normal cell growth\[4,6\]. This is in part attributed to interactions with oncogenes. Moreover, activation of IGF-1R may contribute to tumor angiogenesis by up-regulation of vascular endothelial growth factor (VEGF) expression in certain cancer entities\[5,7-9\]. In the past, different strategies were used to inhibit IGF-1R function, among them monoclonal antibodies and anti-sense RNA directed against the receptor or recombinant IGF binding proteins, and IGF-specific antibodies reducing levels of ligands\[3\]. Thus, targeting the IGF-1R system with small molecule tyrosine kinase inhibitors, such as NVP-AEW541, a novel compound which is 27-fold more selective for IGF-1R than the insulin receptor at the cellular level, may be a new strategy of cancer growth inhibition\[10,11\]. Antineoplastic efficacy of NVP-AEW541 has recently been shown in experimental models of acute myeloid leukemia\[12\], multiple myeloma\[13\], multiple myeloblastoma\[14\], neuroblastoma\[15,16\], medulloblastoma\[17,18\], malignant rhadoblast tumors\[19\], Ewing’s sarcoma\[20\], ovarian\[21\] and breast cancer\[22-25\], mesothelioma\[26\], synovial sarcoma\[27,28\], head and neck squamous cell carcinoma\[29\], adrenocortical tumors\[30\], hepatocellular carcinoma\[31,32\], neuroendocrine gastrointestinal tumors\[33\], gastrointestinal stromal tumors\[34\], colorectal\[35,36,38\], esophageal\[39\], gastric\[40\], and pancreatic cancer\[35,37,39\]. However, little is known about the situation for biliary tract cancer (BTC), a rare tumor with a grim prognosis and limited treatment options. Two recent studies showed expression of IGF-1R and its ligands in gallbladder carcinoma (GBC)\[40\] and cholangiocarcinoma (CC) specimens\[41\]. Therefore, the objectives of the current study were to investigate IGF-1R expression in BTC cell lines and to evaluate the efficacy of in vitro treatment with selective IGF-1R inhibitor NVP-AEW541 alone or in combination with gemcitabine, 5-fluorouracil (5-FU) or Polo-like kinase 1 inhibitor BI 2536, which is currently being investigated in phase II studies including our hospital for the treatment of solid tumors\[42\].

**MATERIALS AND METHODS**

**Drugs and cells**

Seven BTC cell lines; five extrahepatic CC cell lines (EGI-1, TFK-1, CC-SW-1, CC-LP-1, and SK-ChA-1)\[23-25\] and two GBC cell lines (Mz-ChA-1, Mz-ChA-2)\[46\], were examined. All cell lines were cultured in a 37°C incubator with 5%-10% CO₂ in appropriate media, which were changed every 3 d. NVP-AEW541 (targeting IGF-1R) was obtained from Novartis (Basel, Switzerland), dissolved in dimethyl sulfoxide (DMSO) (as 10 mmol/L stock) and stored at -20°C according to manufacturer’s instructions. BI 2536 (targeting Plk-1) was kindly provided by Boehringer (Ingelheim, Germany). Gemcitabine and 5-FU (diluted in 0.9% NaCl) were provided by our hospital pharmacy.

**Inhibition of cell growth**

Cytotoxic effects of drugs alone and in combination were determined by automated cell counting (Casy Cell Counter Model TT; Innovatis AG, Reutlingen, Germany) according to manufacturer’s instructions. Briefly, 2 x 10⁵ cells were seeded in duplicates in T25 flasks with media containing the designated drugs or vehicle control followed by incubation for 3 or 6 d. For the 6 d experiment, medium was changed after 3 d and treatment repeated. At the end of incubation, cells were trypsinized, washed, and analyzed in triplicates by automated cell counting.

**Immunoblotting**

Cell culture monolayers were washed with ice-cold PBS and lysed in flask with a buffer containing Tris-HCl (50 mmol/L, pH 7.4), NP-40 (10 g/L), NaCl (200 mmol/L), sodium-orthovanadate (200 mmol/L), 2-glycerophosphate (1 mmol/L), sodium fluoride (20 mmol/L), DTT (10 mmol/L), PMSF (200 mmol/L) and 0.2% proteinase inhibitor cocktail (Sigma-Aldrich, Munich, Germany) on ice for 30 min. The lysate was then centrifuged at 13 000 r/min for 15 min and proteins in supernatant were quantified by Bradford protein assay (Bio-Rad, Munich, Germany) and stored at -80°C. Next, 50 or 60 μg of cell lysates were separated on SDS-polyacrylamide gels and elec
troblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution [25 g/L dry milk or BSA in TBS-T (10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20)], followed by incubation with the primary antibody at 4°C overnight (50 g/L BSA in TBS-T). The membranes were then washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemiluminescence reaction (SuperSignal West Dura or SuperSignal West Femto, Pierce, Rockford, USA). Monoclonal (mc) β-actin antibody was purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), polyclonal IGF-1R(mAb) from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, USA), mc p-IGF-1R, mc p-p42/44 (p-Erk1/2, p-MAPK), mc p42/44, mc p-AKT, mc AKT, mc p-Stat3, mc Stat3 and mc Bel-αL antibodies were all from Cell Signaling (Cell Signaling Technology, Beverly, USA). Recombinant human IGF-1 was purchased from Biomol (Biomol, Hamburg, Germany).

**Cell cycle analysis**

Cells were seeded in T-25 flasks (3.5 x 10⁵), treated with various concentrations of NVP-AEW541 or vehicle control for 36 h, washed with PBS, trypsinized,
centrifuged, and fixed in ice-cold ethanol with phosphate-buffered saline containing 1 mmol/L EDTA. DNA was labelled with 1:100 diluted propidium iodide after digestion of RNA by RNAse A. Cells were analysed by flow cytometry with a FACSCalibur system (Becton Dickinson, San Diego, USA) and cell cycle profiles were determined using ModFitLT 2.0 for Macintosh (Verity Software House, Topsham, ME, USA). Doublets were excluded by gating for width of fluorescence signal (FL2-W). Each experiment was performed at least in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR) for ligands IGF-1 and IGF-2

Total cell RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) after homogenization with the QIAshredder (Qiagen) according to manufacturer’s instructions. RNA was dissolved in water and quantified at 260 nm with a biophotometer (Eppendorf, Hamburg, Germany); purity was verified by optical density (A) absorption ratio A260 nm/A280 nm between 1.93 and 2.06. Single step quantitative RT-PCR analysis was carried out in the LightCycler system (Roche), primers and fluorochromes were obtained from Qiagen (QuantiTect Primer Assays Hs_IGF1_1_SG and Hs_IGF2_1_SG and SYBR-Green RT-PCR kit) and used according to manufacturer’s instructions. Products of RT-PCR were separated by gel electrophoresis to confirm correct amplification and size. RNA samples extracted from hepatocellular carcinoma tissue and from HepG2 cell line served as positive controls. Water was used to detect primer interactions and GAPDH as housekeeping gene to assure equal loading. Relative gene expression was calculated with REST software tool as used by Pfaffl and Horgan[48].

Caspase-3 assay

Using the caspase-3 colorimetric assay kit (Sigma, Missouri, USA) according to manufacturer’s instructions, caspase-3 activity was measured. Cells were grown, treated, lysed and centrifuged, and supernatants were used. Based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase-3, resulting pNA was measured photometrically at 405 nm after incubation at 37°C and 5% CO2. A pNA calibration curve was used to calculate results.

Statistical analysis

Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean value with SD. Inter-group comparisons were performed with the Student t test. P values less than 0.05 were considered significant.

RESULTS

Inhibition of cell growth

After 3 d of incubation, tested cell lines were sensitive to NVP-AEW541 (mean IC50 = 0.51 ± 0.44 μmol/L) with CC-LP-1 being the most sensitive and Mz-ChA-1 the least sensitive cell line (IC50 values were calculated with linear regression model). Response differed markedly between the group of extrahepatic CC cell lines (mean IC50 = 0.29 ± 0.15 μmol/L) and the group of the two GBC cell lines (mean IC50 = 1.06 ± 0.47 μmol/L) (P < 0.05). Inhibition of cell growth was more pronounced if incubation time was extended to 6 d (treated twice, on days 0 and 4) with a mean IC50 value of 0.22 ± 0.15 μmol/L. Although not statistically significant, there was again a difference in response for the 6 d experiment between the group of extrahepatic CC cell lines (mean IC50 = 0.18 ± 0.06 μmol/L) and the group of the two GBC cell lines (mean IC50 = 0.3 ± 0.32 μmol/L) (Figure 1A and B, Table 1).

Cell lines Mz-ChA-1, showing a weak, and EGI-1, showing an intermediate response to NVP-AEW541, were selected for further studies of drug mechanism, because each of these lines represented one entity of BTC (GBC and extrahepatic CC). Firstly, the in vitro cell growth inhibition experiment was repeated with a broader spectrum of drug doses to determine the correct 3 d IC50 concentration. To verify calculated 3 d IC50 concentrations and to determine the most effective length of incubation, additional experiments with drug incubation times ranging from 1 to 4 d, with 3 d IC50 concentration, were carried out (Figure 1C), which showed best response after 3 d of incubation. Using data from this experiment, replication times for Mz-ChA-1 (68.16 h) and EGI-1 (30.7 h) were calculated. This difference could be one factor influencing response to NVP-AEW541. DMSO, the solvent of NVP-AEW541, had no influence on cell growth when administered alone (data not shown).

Mechanism of drug action

IGF1-R protein was detectable in all seven cell lines by immunoblot, with expression level variation between the different cell lines (Figure 2A). However, there was no correlation between level of IGF1-R protein expression and inhibition of cell growth by NVP-AEW541. For further analysis of drug mechanism, EGI-1 and Mz-ChA-1 cells were used, representing cells with good and little response to the inhibitor, but intermediate IGF-1R protein expression, respectively. Stimulation of cell

Table 1 Inhibition of cell growth by in vitro treatment with NVP-AEW541

| Cell line | IC50 (μmol/L) |
|-----------|---------------|
|           | 3 d           | 6 d           |
| TFK-1     | 0.26          | 0.28          |
| EGI-1     | 0.28          | 0.14          |
| CC-LP-1   | 0.15          | 0.12          |
| CC-SW-1   | 0.54          | 0.20          |
| Sk-ChA-1  | 0.20          | 0.18          |
| Mz-ChA-1  | 1.39          | 0.52          |
| Mz-ChA-2  | 0.73          | 0.07          |
Experiments were performed with Mz-ChA-1 and EGI-1 cells. Treatment with NVP-AEW541 resulted in dephosphorylation in both cell lines. Level of p-p42/44 was already increased in unstimulated EGI-1 cells, but not in Mz-ChA-1 cells. Treatment with NVP-AEW541 resulted in dephosphorylation in Mz-ChA-1 cells, but not in EGI-1 cells. This could be explained by a k-ras mutation (G12D) in the EGI-1 cell line, as already described in the COSMIC catalogue\cite{50} and would result in a consecutively active state of downstream target p42/44, as found by our group. Level of p-Stat-3 was already increased in both unstimulated cell lines. While dephosphorylation of Stat-3 occurred in EGI-1, phosphorylation of Stat-3 remained unchanged during treatment with NVP-AEW541 in Mz-ChA-1 cells. Furthermore, repetition with higher doses of NVP-AEW541 (up to 5 \(\mu\)mol/L) did not show any effect (blot not shown).

Anti-apoptotic protein Bcl-xL was significantly reduced after treatment with NVP-AEW541 in Mz-ChA-1 cells, but we could not determine any significant change of expression in EGI-1 cells after treatment. Treatment with higher doses of NVP-AEW541 did not reveal greater effects (blots not shown). Staining with \(\beta\)-actin antibody confirmed equal protein loading in all immunoblots.

Cell cycle analysis of cell lines EGI-1 and Mz-ChA-1, treated with NVP-AEW541 for 36 h, showed an increase of G0/G1 fraction, possibly caused by G1/S-checkpoint arrest. This arrest was more pronounced when the dose was increased (Figure 3A and B). Analysis of flow cytometry sub-G1 fraction showed no significant apoptosis for NVP-AEW541 concentrations below 1 \(\mu\)mol/L, but marked apoptosis if NVP-AEW541 concentration was increased above 1 \(\mu\)mol/L (Figure 3C). Treatment with 5 \(\mu\)mol/L resulted in marked apoptosis, influencing cell cycle proportions. There was no significant change in caspase-3 activity induced by treatment with NVP-AEW541 (data not shown).

**RT-PCR analysis of IGF-1R ligands IGF-1 and IGF-2**

Semiquantitative RT-PCR revealed expression of ligands IGF-1 and IGF-2 in selected MzChA-1 and EGI-1 cells, suggesting an autocrine loop of IGF-1R activation. Samples of human hepatocellular carcinoma tissue served as a positive control. There was no significant change in expression levels of either IGF-1R ligand induced by treatment with NVP-AEW541 for 3 d (Figure 4).

**In vitro combination of NVP-AEW541 with gemcitabine, 5-FU or BI 2536**

To evaluate efficacy of NVP-AEW541 in combination with commonly used chemotherapeutics for the treatment of BTC, further in vitro experiments were performed. NVP-AEW541 at IC\(_{50}\) concentration (0.8 \(\mu\)mol/L for Mz-ChA-1 and 0.20 \(\mu\)mol/L for EGI-1) was combined with increasing concentrations of gemcitabine or 5-FU over 3 d (Figure 5). Assuming that the drugs do not directly interact and using the model of effect multiplication postulated by Berenbaum\cite{49},
Figure 2  Mechanism of NVP-AEW541 drug action. A: Protein expression of IGF-1R in tested human biliary tract cancer cell lines was determined by immunoblot; B: p-IGF-1R, IGF-1R, p-AKT, AKT, p-p42/44, p42/44, p-Stat-3, Stat-3, and Bcl-xL protein levels were examined by immunoblot for selected cell lines EGI-1 and Mz-ChA-1. After 24 h of starvation, cell lines EGI-1 and Mz-ChA-1 were treated with NVP-AEW541 (12 h prior to lysis) and stimulated with ligand IGF-1 (30 min prior to lysis). β-actin served as loading control in all experiments. Densitometry was performed to analyze results which are shown relative to lane 2 as standard.
calculated values representing synergistic effects were compared to measured values. Even though IC\textsubscript{50} doses of NVP-AEW541 for both cell lines are different, gemcitabine showed synergistic effects at low concentrations for both tested cell lines (Figure 5A and B), whereas combinations of NVP-AEW541 with 5-FU showed only additive effects (Figure 5C and D). In addition, new small-molecule Plk-1 inhibitor BI 2536 was tested in combination with NVP-AEW541. This compound was available to us due to our participation in a phase II study for the treatment of solid tumors. Similar to the combination with 5-FU, the combination with BI 2536 resulted only in additive effects (Figure 5E and F).

**DISCUSSION**

Non-resectable BTC is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy. It is therefore essential to search for new therapeutical approaches\textsuperscript{[51]}. In a recent study, analyzing tumor specimens from 57 patients with GBC, a strong immunoreactivity for IGF-1R was shown in 95%. IGF-1 and IGF-2 expression was detected in 45% and 25% of specimens, respectively\textsuperscript{[40]}. Another study found IGF-1 and IGF-1R expression in all 18 examined biopsy samples of intrahepatic CC, whereas cholangiocytes of intrahepatic bile ducts of normal human liver were all negative\textsuperscript{[41]}. Results of our own in vitro experiments support the co-expression of IGF receptor and its ligands IGF-1 and IGF-2. This co-expression, which is not a physiological state, leads to the assumption of an autocrine loop, rendering the IGF system a possible target for specific anti-cancer therapy of BTC.

Different approaches to inhibit IGF-1/-2 effects, at receptor or downstream levels, have already shown promising results in other studies. While IGF-1 neutralizing antibodies or IGF-binding proteins reduce ligand levels, strategies directed against IGF-1R employ receptor-specific antibodies which are mainly effective through degradation and downregulation of the receptor after binding, i.e. siRNAs, and small molecule tyrosine kinase inhibitors\textsuperscript{[5]}. Several IGF-1R specific fully humanized monoclonal antibodies are currently already in phase II clinical trials (MK-0646 by Merck Oncology for solid tumors; AMG 479 by Amgen Oncology for Ewing’s sarcoma, breast and pancreatic cancer; CP-751,871 by Pfizer Oncology for prostate cancer and NSCLC).

Our results for the small molecule tyrosine kinase inhibitor NVP-AEW541 showed a potent growth inhibition of BTC cell lines, supported by the low 3 d IC\textsubscript{50}
doses we determined. Although our results show different responses toward treatment between the groups of CC and GBC cell lines, comparable results have only been reported for acute myeloid leukemia [12], medulloblastoma [17], neuroblastoma [15,16], Ewing’s sarcoma [21], fibrosarcoma cells [11], and breast cancer cell line MCF-7[25] whereas IC50 concentrations were much higher for ovarian cancer [22], mesothelioma [26], osteosarcoma [21], synovial sarcoma [27], neuroendocrine gastrointestinal tumor [33], gastrointestinal stromal tumor [34], pancreatic cancer [39], hepatocellular carcinoma [31], and colorectal cancer cells [38] (up to 50 μmol/L). However, the exact molecular mechanisms for these differences in response are still unclear. One factor might be the level of IRS-1 [25]; other factors might be mutations (e.g. of k-ras), and differences in replication time of cells.

Small molecule inhibitors act only by reducing IGF-1R activation and seem not to influence receptor expression. We were able to show the suppressive effect of NVP-AEW541 on phosphorylation of IGF-1R, while whole IGF-1R protein expression remained stable. At the same time, treatment with NVP-AEW541 did not result in significant upregulation of IGF-1 and IGF-2 levels. These results support treatment efficacy of NVP-AEW541.

Dephosphorylating effects on downstream targets p42/44 (thus reducing rate of proliferation) and AKT (thus increasing rate of apoptosis) were consistent with

Figure 5 In vitro treatment with drug combinations of NVP-AEW541 and gemcitabine, 5-fluorouracil (5-FU) or BI 2536. Selected cell lines EGI-1 and Mz-ChA-1 were incubated with increasing concentrations of gemcitabine (A, B), 5-FU (C, D) or BI 2536 (E, F) alone (black circles) and in combination with fixed IC20 concentration of NVP-AEW541 (black triangles). The shaded area represents a possible drug synergism calculated according to the model of Berenbaum[50]; error bars represent SD (n = 3).
previous studies (with the exception of consecutively activated p42/44 in cell line EGI-1)\cite{11,12,13,21,26,34,35,39}. The JAK/STAT (signal transducer and activator of transcription) signal cascade is another important pathway in oncogenesis in general\cite{38}, and in the pathogenesis of CC in particular, since it has already been shown that upregulation of anti-apoptotic Mcl-1 (myeloid cell leukemia-1) by interleukin-6 depends on Stat-3 activation\cite{33,34}. Moreover, RACK1 (receptor for activated C kinase 1) may be the adaptor for IGF-1R-mediated Stat-3 activation\cite{39}. Moser et al\cite{39} recently reported a decrease of Stat-3 phosphorylation after blockade of IGF-1R in pancreatic cell line HPAF-Ⅱ. In our own in vitro experiments, this effect was only seen in cell line EGI-1, but not in cell line MzChA-1.

Further results of our experiments showed that effects of NVP-AEW541 seem to be based mainly on an inhibition of cell growth, at least at low concentrations, while only high doses seem to trigger apoptosis. Although we could not detect any apoptosis by caspase-3 assay, levels of anti-apoptotic protein Bcl-xL decreased after treatment of MzChA-1 cells. In flow cytometry, the rate of apoptotic cells (sub-G1 peak) increased markedly at drug concentrations above 1 μmol/L.

Another possible anti-cancer mechanism of NVP-AEW541, which was not examined in our experiments, might be the inhibition of angiogenesis, since it has been demonstrated that IGF-1 can induce expression of VEGF\cite{39} and that NVP-AEW541 significantly reduces vascularization and VEGF expression in an in vivo mouse model for pancreatic cancer\cite{39}. In addition, it has recently been shown that IGF-1R blockade reduces the invasiveness of gastrointestinal cancers via blocking production of matrilysin\cite{39}.

Regarding the obviously different response rates of extrapathic CC cell line EGI-1 and GBC cell line Mz-ChA-1, it is not easy to find a simple explanation. While a longer replication time and resistance to Stat-3 dephosphorylation would predict the lower response of Mz-ChA-1 cells to NVP-AEW541, it is unclear why resistance to dephosphorylation of p-p42/44 via k-ras mutation and resistance to Bel-xL downregulation did not induce a lower response of EGI-1 cells to NVP-AEW541. Perhaps these factors have a different impact of contribution to resistance.

A possible side effect of NVP-AEW541 could be induction of diabetes due to the high homology of the kinase region of IGF-1R and the insulin receptor. While in vitro kinase assays showed a 27-fold selectivity of NVP-AEW541 towards IGF-1R\cite{61}, a recent in vivo study found neither an increase of blood glucose level nor other side effects in treated animals\cite{39}.

Since resistance against anti-cancer drugs evolves rapidly, a combination of different approaches seems necessary. The combined drugs should ideally tackle the cancer cells in different cell phases and use different modes of action. Gemcitabine and 5-FU are currently used as chemotherapy for BTC\cite{39}. Gemcitabine is a nucleoside analogue that is utilized instead of cytidine during DNA replication, leading to premature chain termination and subsequent apoptosis. While 5-FU is principally also a nucleoside analogue, its main effects are exerted through inhibition of thymidylate synthase and hence reduction of thymidine necessary for DNA replication. Both drugs affect cells mainly during S phase, while our flow cytometry experiments showed that treatment with NVP-AEW541 led to G1 phase arrest, leading to synergistic effects in combination with gemcitabine. In contrast, the combination with 5-FU was less effective, possibly derived from the facts that 5-FU in general seems to be less effective than gemcitabine in the treatment of BTC\cite{39} and that 5-FU is more effective at reduced doses for extended periods of exposure\cite{39}. Additionally, we tested the combination of NVP-AEW541 with BI 2536. Polo-like kinases are increasingly recognized as key regulators of mitosis, meiosis and cytokinesis\cite{57} and have been implicated in the transformation of human cells\cite{58}. BI 2536, a novel selective inhibitor of Plik-1, inhibits tumor growth in vitro\cite{59}. The lack of synergistic effects of BI 2536 in combination with NVP-AEW541 may be attributed to the assumption that BI 2536 affects the M phase of the cell cycle, which occurs less frequently because of NVP-AEW541-mediated G1 arrest. Another promising combination therapy may be dual inhibition of IGF-1R and FAK (focal adhesion kinase) as has already been shown for the novel single small molecule inhibitor TAE226, which targets specifically both FAK and IGFR-1\cite{60}. Further suggested combination partners are trastuzumab (HER-2 inhibitor) in HER-2 positive breast cancer\cite{29}, erlotinib (EGF-R inhibitor)\cite{38}, ICR62 (anti-EGF-R monoclonal antibody)\cite{57}, and mammalian target of rapamycin inhibitor RAD001\cite{57}.

In summary, our findings suggest that NVP-AEW541 is active against BTC in vitro. In addition, the compound potentiated the efficacy of gemcitabine. Based on this data, further preclinical and clinical evaluation of this new drug for the treatment of BTC is recommended.

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**COMMENTS**

**Background**

Carcinomas of the biliary tree are rare tumors of the gastrointestinal tract with a rising incidence worldwide for intrapathic cholangiocarcinoma (CC) in recent years. At present, complete resection is the only potentially curative therapy, but most patients present with already advanced disease. In the palliative setting, non-resectable biliary tract cancer (BTC) is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy.

**Research frontiers**

Receptor tyrosine kinase inhibitors are currently under preclinical and clinical evaluation as new treatment options.
Innovations and breakthroughs

After several years of preclinical research, the first clinical study data are now available for this tumor entity. Inhibitors of the epidermal growth factor receptor family, such as erlotinib, cetuximab, and lapatinib were recently investigated. Bortezomib, an inhibitor of the proteasome, matinib mesylate, an inhibitor of c-kit, bevacizumab, an inhibitor of vascular endothelial growth factor (VEGF); and Sorafenib (BAY 43-9006), a multiple kinase inhibitor that blocks not only receptor tyrosine kinases but also serine/threonine kinases along the RAS/RAF/MEK/ERK pathway, have also been studied. Although early evidence of antitumor activity was seen, the results are still preliminary and require further investigations.

Applications

The aim of the authors’ study was to investigate the in vitro treatment with NVP-AEW541, a small molecule inhibitor of insulin-like growth factor-1 receptor (IGF-1R), in BTC. Their findings suggested that cell growth suppression was successful in seven human BTC cell lines. Combined with gemcitabine, NVP-AEW541 exerted synergistic effects, particularly at low concentrations, while effects of combinations with 5-fluorouracil or Polo-like kinase 1 inhibitor BI 2536 were only additive.

Terminology

The IGF-1R system has emerged as an interesting target for cancer therapy, as it represents an important promoter of tumor transformation and survival of malignant cells, but is only partially involved in normal cell growth. This is in part attributed to interactions with oncogenes. Moreover, activation of IGF-1R may contribute to tumor angiogenesis by up-regulation of VEGF expression in certain cancer entities. In the past, different strategies were used to inhibit IGF-1R function, among them monoclonal antibodies and anti-sense RNA directed at receptor. The aim of the authors' study was to investigate the antitumor effect on neuroblastoma cells in vitro and in vivo. Cancer Chemother Pharmacol 2006; 12: 6772-6780

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The strength of this manuscript is that it characterized a highly-resistant BTC cell line in comparison with an extrahepatic CC cell line. Also, to some extent, the mechanism leading to the resistance was examined.

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