Validation of VX2 as a Hepatocellular Carcinoma Model: Comparison of the Molecular Reaction of VX2 and HepG2 Tumor Cells to Sorafenib *In Vitro*

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Abstract. As there is currently no superior hepatocellular carcinoma (HCC) model with percutaneous vascular access for transarterial treatments available, the VX2 rabbit model is frequently used for *in vivo* investigations on liver carcinoma. However, the VX2 cell line was derived from a virus-induced skin papilloma that can form carcinosarcoma in liver of rabbits and the transferability of obtained results to HCC treatment remains open. Here we compared the most frequently investigated human HCC model cell line, HepG2, with VX2 cells *in vitro* in terms of sensitivity towards the broad specificity kinase inhibitor sorafenib and responsiveness to the addition of platelet-derived growth factor AB (PDGF-AB), vascular endothelial growth factor (VEGF) and hepatic growth factor (HGF), as well as insulin and interleukin-1 β (IL1 β). Phosphorylation of protein kinase B (AKT) the mitogen-activated protein kinases (MAPKs) p38 and p42/44 (extracellular signal-regulated kinase, ERK1/2) and inhibitor of kappa light chain gene enhancer alpha (I κ B α) was determined by western blotting as these events are associated with early signaling cascades. Additionally, the inhibition of phosphorylation under sorafenib treatment was investigated. Sorafenib was equally toxic to both cell lines, but only in HepG2 was activation of caspase 3/7 activity, as a sign of apoptosis, observed. VX2 cells exhibited generally more intense phosphorylation signals in response to the growth

factors and also serum. In contrast to VX2, HepG2 cells showed no response to PDGF-AB or VEGF as determined by kinase phosphorylation. In both cell lines, sorafenib inhibited growth factor-induced phosphorylation of ERK and p38-MAPK. AKT phosphorylation was only inhibited in VX2 cells and I κ B α phosphorylation was not influenced by this kinase inhibitor in either cell type. Taken together, the two cellular models for HCC share several features related to sorafenib application, but differed in their responsiveness towards growth factors. Therefore, results obtained with the VX2 model cannot be extended to human HCC without appropriate caution.

The treatment of hepatocellular carcinoma (HCC) remains a challenge: the incidence is rising worldwide and the disease is still frequently diagnosed in an advanced stage at which curative treatment cannot be provided. Potentially curative liver transplantation is restricted to a minor proportion of these patients. Furthermore, long-term survival from resection or local ablation by radiofrequency can only be performed at very early and early stages of disease [according to the Barcelona Clinic Liver Cancer (BCLC) scheme] (1-4). Transarterial chemoembolisation (TACE) is accepted as the therapy of choice in selected patients within the intermediate disease stage (BCLC B) or as bridging treatment for patients on the waiting list for transplantation (1, 2, 5-7). When TACE is contraindicated, as well as in advanced disease, systemic therapy with sorafenib, a multikinase inhibitor, is the only available effective treatment (8). Radioembolization with ⁹⁰Y as alternative or additive treatment at this stage of disease is currently under investigation in large phase III trials (9).

Through its embolic effect, TACE may induce the release of angiogenic factors, which in turn may promote growth of surviving tumor cells leading to tumor recurrence and

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metastatic spread. Therefore, combining TACE with systemic sorafenib treatment is an attractive concept (10, 11). However, clinical data on this issue so far are inconclusive (12, 13). In order to enhance the adjunctive effect of sorafenib combined with TACE and to reduce the side-effects of systemic sorafenib therapy, local application of sorafenib into the HCC, together with embolic material, is proposed and under initial preclinical research, with promising results (14-18).

The common preclinical TACE model is the VX2 tumor-bearing rabbit model (14, 15, 17, 19, 20). VX2 is a virus-induced papilloma transforming to an aggressive anaplastic carcinosarcoma of the rabbit and unfortunately no hepatoma model for the rabbit (as for rats or mice) exists (21, 22). However, due to percutaneous vascular accessibility in the rabbit and a pattern of vascularization similar to HCC, VX2 is a widely accepted model of HCC for TACE treatment (16, 20, 23). It remains unclear whether data drawn from the VX2 model can be adopted for HCC treatment, especially if complex and specifically acting substances such as sorafenib are used. Data on *in vitro* comparison of VX2 to hepatoma cells is very scarce. In one study the glucose metabolism of VX2 was compared to that of hepatoma cells (AS-30D), showing a comparable pattern of glucose utilization and metabolism (24).

Recently, a comparison of the sensitivity of the VX2 and HepG2 cell lines to chemotherapeutic agents was published (25). It was found that sensitivity to doxorubicin, mitomycin-C, sunitinib, sorafenib and lapatinib was similar, whereas the toxicity of platins and irinotecan in particular clearly differed between the cell lines.

To our knowledge, there is no study focusing on the molecular reaction of VX2 cells to sorafenib. In order to further elucidate if the VX2 model can serve as a valuable HCC paradigm when sorafenib is applied, we sought to compare the molecular response of VX2 cells to those of HepG2 cells under sorafenib exposure *in vitro*. As study parameters, cell viability/proliferation, apoptosis (as caspase 3/7 activity) and phosphorylation of kinases representing the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) pathway [inhibitor of kappa light chain gene enhancer alpha (I κ B α)], proliferative responses [protein kinase B (AKT) and extracellular signal-regulated kinases (ERK1/2)] were chosen (26).

Materials and Methods

Cell lines, growth factors and sorafenib. VX2 cells were obtained from IAZ (Munich, Germany) and the HepG2 cell line was obtained from the American Type Tissue Collection (ATCC) stock center. Both cell lines were maintained in RPMI-1640 medium supplemented with fetal calf serum (FCS; 10%) and penicillin/streptomycin (Biochrom, Berlin, Germany) at 37°C in a humidified cell culture incubator (Heraeus, Hanau, Germany) in

5% CO₂. Cells were supplied with fresh medium every 3 days and transferred weekly after detachment with trypsin/EDTA (Biochrom, Berlin, Germany). All cell culture dishes, plates and flasks were from TPP (Trasadingen, Switzerland). Hepatic growth factor (HGF), vascular endothelial growth factor (VEGF) and interleukin-1 β (IL1 β) were from Miltenyi-Biotec (Bergisch-Gladbach, Germany); insulin was from Sigma Aldrich (Taufkirchen, Germany); platelet-derived growth factor (27) was from Professor Hoppe (Würzburg, Germany); sorafenib tosylate was provided by Bayer (Berlin, Germany).

Cell stimulation and western blotting. For stimulation with different growth factors, cells were seeded into 24-well plates and grown to confluence. The medium was then removed, the cells washed with serum-free medium and further incubated in serum-free (HepG2) or serum-reduced (0.5%; VX2) medium for 24 h. Before stimulation, either sorafenib dissolved in dimethyl sulfoxide (DMSO) or solvent (final DMSO concentration 0.1%) was added for 90 minutes preincubation. Effectors were then added (10 ng/ml IL1 β , 20 ng/ml VEGF, 100 nM insulin, 10 ng/ml PDGF-AB, 2.5 to 100 ng/ml HGF) and after 10 minutes, the medium was aspirated and the cells lysed with 50 μ l SDS-lysis buffer [50 mM TRIS/Cl pH 6.8, 2% sodium dodecyl sulfate (SDS), protease and phosphatase inhibitors (1/100; Sigma-Aldrich)]. The plates were stored at -20°C until lysates were transferred into tubes and denaturing sample buffer was added. SDS-polyacrylamide electrophoresis was performed on 12% polyacrylamide (PAA) gels and proteins were transferred to nitrocellulose filters (0.45 μ m; Whatman-GE-Life Sciences, via VWR, Darmstadt, Germany) by tank blotting (BioRad, Munich, Germany) as previously described (28, 29). Blots were stained with Ponceau Red (Sigma-Aldrich) to control transfer quality and then blocked in TBS (50 mM TRIS/Cl pH 7.4 150 mM NaCl), containing 2% bovine serum albumin (BSA; Sigma-Aldrich), NP-40 (0.2%; Applichem, Darmstadt, Germany) and sodium azide (0.03%) for 1 h at room temperature. Blots were incubated with primary antibodies in blocking buffer overnight at 4°C. Antibodies directed against the phosphorylated proteins (AKT, ERK1/2 and I κ B α) were from Cell Signaling (via NEB, Frankfurt, Germany), β -actin monoclonal mouse antibody was obtained from Sigma-Aldrich. After washing three times with TBS (0.2% BSA; 0.2% NP-40), horseradish peroxidase conjugated secondary antibody (DAKO, Hamburg, Germany) was applied for 1 h at room temperature in washing buffer. After three further washes, detection was performed by chemoluminescence with ECL Plus reagent (Millipore, Darmstadt, Germany) in a GeneGnome imaging system (Syngene, Cambridge, UK). Signals were quantified by using ImageJ (30) and normalized to the β -actin signal.

Cell viability and caspase assay. Cell viability and proliferation as well as caspase assays were performed as described previously (29). For cell viability assays, cells were seeded into 24-well plates (Greiner, Frickenhausen, Germany) at about 30% confluence in full medium. The next day, sorafenib or solvent was added. After 72 h, resazurin (Sigma-Aldrich) was added (10 μ g/ml final concentration) and the cells incubated for 30 to 120 min at 37°C, depending on color development. Then the fluorescence of 100 μ l supernatant was determined in a 96-well plate at wavelengths 525/580-640 nm (excitation/emission, fluorescence module 'green') in a Glomax microtitre plate reader (Promega, Mannheim, Germany). Data were normalized to the fluorescence signal obtained for control solvent-treated cells.

Figure 1. A: Toxicity of sorafenib. Cells were grown in full medium supplemented with sorafenib or solvent (dimethyl sulfoxide) for 72 h. Viability/proliferation was determined by the resazurin assay. B: Activation of caspase 3/7 as determined by a luminogenic enzyme assay. Cells were incubated for the indicated time with sorafenib, then lysed and the caspase 3/7 activity determined as described in the Materials and Methods section. *Significantly different between the two cell lines ($p < 0.05$).

For caspase 3/7 determinations, cells were seeded into 96-well plates at about 50% confluence. The next day sorafenib was added and the caspase activity was measured at 0, 2, 4 and 6 h using the caspase 3/7-glo luminescence assay system (Promega). Luminescence was read in a Glomax multidetection system (Promega). Data were expressed relative to control treatments for each cell line.

Statistical calculation. Experiments were at least performed three times with each treatment in duplicate. All calculations were performed with SPSS vers. 22 (IBM, Ehningen, Germany). Data are presented as mean $\pm$ standard error (SEM). Tests for significant differences of means of the results for the two cell lines were performed using ANOVA with *post hoc* Tamhane T2 test. A value of $p < 0.05$ was considered significant.

Results

Toxicity of sorafenib. Firstly, the toxicity of sorafenib towards the two cell lines was determined. According to a previously described method (31), we incubated the cells in full medium with and without sorafenib or DMSO for 72 h. Viability together with proliferation was determined by this approach. HepG2 and VX2 cells both exhibited an half-maximal effective concentration (EC_{50}) value of about 1.8 μ M (Figure 1A). To examine the type of cell death, we performed additional caspase 3/7 assays in sorafenib-treated cells. Only HepG2 cells responded significantly by activation of this effector caspase of responded apoptosis (Figure 1B).

Cell response to different stimuli. In the next set of experiments, we stimulated the two cell lines with several agents, including serum, growth factors and IL1 β . For this,

the cells were grown to confluence and subjected to serum starvation for 24 h. Such resting cell populations exhibit low kinase activities, and thus are able to respond to stimuli without interference from serum and proliferation. As the VX2 cell line did not tolerate complete serum removal, the serum starvation was performed with the lowest tolerable amount of FCS, namely 0.5%. Firstly, we determined the optimal inhibitory concentration of sorafenib for ERK phosphorylation in unstimulated and serum-stimulated cells (Figure 2). Significant inhibition was observed at a sorafenib concentration of 2.5 μ M and higher. HepG2 cells seemed slightly less sensitive, but this was not statistically significant. We therefore continued with 5 μ M sorafenib for the investigation of this inhibitor on growth factor responses. This concentration resulted in 60% and 40% reduction of ERK phosphorylation in VX2 and HepG2, respectively (Figure 2).

We observed that VX2 cells generally responded more strongly to most of the stimuli provided, with rapid phosphorylation of AKT, p38 MAPK and ERK (p42/44 MAPK) (Figure 3). In detail, ERK and p38 MAPK phosphorylation was induced with FCS, PDGF-AB, insulin, IL1 β and VEGF. Additionally, strong phosphorylation of AKT was observed in response to serum and PDGF-AB. IL1 β also caused phosphorylation of I κ B α , indicating activation of the NF κ B pathway. ERK, AKT and p38 phosphorylation was nearly completely inhibited by sorafenib (5 μ M). No effect of sorafenib on I κ B α phosphorylation was observed. HepG2 cells showed only a weak response to serum, without p38 phosphorylation, and

Figure 2. Dose response of extracellular signal-regulated kinase phosphorylation (pERK) to sorafenib (SORA). Cells were serum-starved for 24 h and then preincubated with different sorafenib concentrations as indicated for 1 h. Cells were then stimulated by adding 5% fetal calf serum (FCS) for 10 min and proteins were analyzed by western blotting as described in Material and Methods. Experiments were performed in triplicate and a representative result is shown. Intensity of pERK signals was normalized to the actin loading control and control cell samples set to 1. These dose-response data are quantitatively shown as a bar graph (right).

Figure 3. Signaling-associated phosphorylation of protein kinases. Cells were stimulated for 10 min after 24-h serum starvation as described in the Materials and Methods. Protein phosphorylation was detected by western blotting. Experiments were performed in triplicate with one representative result shown. Concentration of stimulatory agents was 5% fetal calf serum (FCS), 10 ng/ml platelet-derived growth factor AB (PDGF-AB), 100 nM insulin, 10 ng/ml interleukin-1β (IL1β) and 20 ng/ml vascular endothelial growth factor (VEGF).

apparently no response to PDGF-AB and VEGF. Insulin, in contrast, caused a much stronger response than in VX2 cells, especially of AKT phosphorylation. Whereas sorafenib inhibited ERK and p38 phosphorylation, no effect on AKT was seen.

For HGF, similar responses were seen in both cell lines, however, VX2 was again more sensitive, as shown by a dose-response experiment (Figure 4).

Discussion

In this study, we compared two frequently used models for HCC: the VX2 rabbit model, which is often used for *in vivo* studies, and the HepG2 cell line, which is regularly used for *in vitro* studies. We focused on the kinase inhibitor sorafenib,

as this compound is an important drug for treatment of patients with HCC. Furthermore, it has become the focus of interest as it was recently applied in combination with transarterial chemoembolization (TACE) in preclinical series (14-18).

The VX2 cell model is currently the only rodent model system with percutaneous transarterial accessibility for studying locoregional treatment of HCC *in vivo*. However, its origin from skin papilloma suggests that these carcinosarcoma cells do not behave exactly as would be expected for liver cell carcinomas. However, others have pointed out that VX2 cells are as sensitive as HepG2 cells to a variety of chemotherapeutic agents, although some significant differences were found (23, 25). Indeed, here we also determined a practically identical EC₅₀ for sorafenib

Figure 4. Dose response of kinase phosphorylation of VX2 and HepG2 cells to hepatic growth factor (HGF). Cells were grown to confluency, subjected to serum starvation for 24 h and then stimulated for 10 min with the indicated factors. Dimethyl sulfoxide was used as solvent control for sorafenib treatment.

toxicity for both cell types, and most of the growth factors used evoked comparable signaling responses in both.

Nevertheless, only HepG2 cells activated caspase 3/7, indicating apoptosis upon sorafenib exposure, whereas VX2 seems to execute cell death by another mechanism that we did not further determine in this study but which others suggest as being through autophagy (32).

The EC_{50} values for survival have been previously determined and our data (1.8 μ M) are within the published range. Liu *et al.* determined an EC_{50} value of 4.5 μ M for HepG2 cells, also after 72 h exposure, but used an luminescent ATP assay for determination of cell viability (26). Pascale *et al.* found higher values of 10.4 for VX2 and 9.0 μ M for HepG2 after 72-h incubation with sorafenib and determined the cell number indirectly by measuring total cellular protein (25). We assume that these differences can be attributed to different experimental approaches, including not only the method to determine proliferation and viability, but also different media compositions and serum batches.

An important feature for a cancer cell in its niche is the possibility to respond to various proliferative or inflammatory factors provided by the microenvironment or *via* the bloodstream. The mechanism of sorafenib as a broad-specificity kinase inhibitor is to block such signals in order to inhibit proliferation or cause cell death. Earlier publications have often focused on activation of the ERK pathway by HGF or serum factors (26) and inhibition of ERK phosphorylation by sorafenib; we herein intended to extend these data to other factors important for HCC and additional signaling pathways. Our growth factor set contained PDGF-AB, VEGF, IL1 β , insulin and HGF. PDGF-AB is secreted by HCC cells and responsible for their growth (33). HCC is an inflammation-associated cancer, therefore IL1 β signaling is important for tumorigenesis by *e.g.* up-regulating gankyrin (34). Liver

cells are typical targets for insulin and HCC risk is higher in insulin-resistant diabetic patients (35, 36). VEGF is well known for its effects on vascularization of tumors. In patients with HCC, the *VEGFA* gene is often amplified, which triggers secretion of HGF. Patients with HCC with *VEGFA* gene amplification are reportedly especially sensitive to sorafenib (37). Last but not least, HGF is a multifunctional cytokine which modifies proliferation, motility and morphogenesis of cells by its interaction with its receptor also known as cellular mesenchymal–epithelial transition (cMET) (38).

With stimulated and unstimulated cells, we determined the concentration of sorafenib which effectively blocked ERK phosphorylation. This concentration was comparable in both cell lines (5 μ M), which was consistent with the also virtually identical effective toxic concentration of the drug.

Although the EC_{50} values for sorafenib *in vitro* have been determined to be in the nanomolar range *i.e.* 4 to 90 nM against rearranged during transfection (RET) rat fibrosarcoma-1 (cRAF) and the VEGFR, working concentrations of sorafenib in cell cultures were in the low micromolar range (39), which our data are consistent with.

The responsiveness to growth factors reflects the expression of the respective receptors and the sensitivity of the coupled signaling cascades. HepG2 cells showed no detectable response to PDGF-AB and VEGF. When comparing the responses of the cell lines, we observed a generally higher sensitivity of VX2 for most factors. Interestingly, VX2 also very sensitively responded to HGF but in contrast to HepG2 cells, the response to insulin was rather weak. The latter result is consistent with the importance of insulin signaling for liver cells.

In VX2 cells, sorafenib was able to block growth factor-induced phosphorylation of AKT, ERK and p38 MAPK regardless of the stimulant. In contrast, sorafenib was ineffective in blocking insulin- and FCS-induced AKT

phosphorylation in HepG2 cells. In both cell lines, no reduction of IL1 β -induced phosphorylation of I κ B α and p65 (data not shown) was observed. This was expected as these signals are upstream of ERK and p38 in the IL1 β receptor cascade. Interestingly, IL1 β was among the strongest inducers of p38-MAPK and ERK phosphorylation in HepG2 cells, and these responses were effectively diminished by sorafenib in both HepG2 and in VX2 cells.

These *in vitro* data strongly suggest an important role of inflammatory signaling *via* Toll-like receptors such as the IL1 β receptor for HCC. Indeed, one downstream kinase of the IL1 β receptor, interleukin receptor-associated kinase 1 (IRAK1) is up-regulated in many HCCs and cell lines derived from HCCs (34, 40), suggesting that interleukin signaling may be a target for HCC therapy. As sorafenib effectively inhibits downstream activation of ERK and p38 by IL1 β , this might contribute to the effects of this drug on HCC.

Taken together, we demonstrated several common features of the VX2 and HepG2 cellular models, justifying the use of VX2 cells in rabbit *in vivo* studies. However, as distinct differences were also observed, the results obtained in such investigations should always be interpreted with care.

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