Amino Acids (2014) 46:969–976
DOI 10.1007/s00726-013-1662-2

ORIGINAL ARTICLE

Glycine inhibits angiogenic signaling in human hepatocellular carcinoma cells

Helge Bruns · Marius Petrulionis · Daniel Schultze · Mohammed Al Saeedi · Shibo Lin · Kenya Yamanaka · Marius Ambrazevičius · Kęstutis Strupas · Peter Schemmer

Received: 15 September 2013 / Accepted: 29 December 2013 / Published online: 4 January 2014
© Springer-Verlag Wien 2014

Abstract Hepatocellular carcinoma (HCC) is a highly vascularized tumor with limited susceptibility to chemotherapy. Modern targeted therapies are aimed at specific properties of this neoplasm. Glycine is a simple non-essential amino acid with potential antiangiogenic effects. In this study, the amino acid's effect on angiogenic signaling in an in vitro model of HCC was evaluated. HepG2 and Huh7 cells were treated with glycine-free DMEM supplemented with 0, 0.01, 0.1, 1.0, 2.0, 5.0 and 10 mM glycine. The direct effects of glycine on the viability of HCC cells were monitored using MTT assay. To detect angiogenic signaling, mRNA and protein levels of vascular endothelial growth factor (VEGF-A) were measured using RT-PCR and Western Blot assays. To determine whether or not glycine receptors (GlyR) played a significant role, the specific antagonist, strychnine, was used as a direct inhibitor. Western Blotting was performed to show the presence of GlyR. While there was no direct pro- or anti-proliferative effect of either glycine or strychnine in both cell lines, glycine was shown to significantly decrease VEGF-A expression on mRNA and protein level up to 63 % in both cell lines. This effect was blunted by the presence of strychnine. GlyR was also identified in both cell lines. Glycine decreases GlyR-dependent, VEGF-A-mediated, angiogenic signaling in human HCC and thus might be a promising additive to chemotherapy treatment strategies for highly vascularized tumors.

Keywords Glycine · Hepatocellular carcinoma · Angiogenesis

Introduction

Hepatocellular carcinoma (HCC) is the most common histological type of primary liver cancer and the third leading cause for cancer-related deaths worldwide (El-Serag and Rudolph 2007). For most patients, no curative treatment option is available. In addition to surgical therapies and interventional strategies with curative palliative treatment concepts, multimodal approaches that extend survival exist (Sauer et al. 2005; Mazzaferro et al. 2011; Hoffmann and Schmidt 2009; Lubienski et al. 2004; de Lope et al. 2012; Hoffmann et al. 2008). Conventional chemotherapy only marginally affects survival in HCC patients, thus therapies targeting specific molecular pathways have been introduced to make up for the inadequacies of existing therapies (Wei et al. 2013; Cao et al. 2012). Since HCCs are highly vascularized, angiogenesis is considered to be one of the main targets for effective HCC therapy (Bishayee and Darvesh 2012). Current chemotherapeutical agents for HCC include the antiangiogenic multikinase inhibitors as well as mTOR inhibitors (Di Marco et al. 2013; Kelley and Venook 2013; Buitrago-Molina and Vogel 2012; Shiah et al. 2013; Zhou et al.

H. Bruns and M. Petrulionis have contributed equally to this article.

H. Bruns · M. Petrulionis · D. Schultze · M. Al Saeedi · S. Lin · K. Yamanaka · M. Ambrazevičius · P. Schemmer
Department of General and Transplant Surgery, Ruprecht-Karls-University Heidelberg, Heidelberg, Germany

M. Petrulionis · M. Ambrazevičius · K. Strupas
Center of Abdominal Surgery, Vilnius University Hospital Santariskiu Klinikos, Vilnius, Lithuania

P. Schemmer (✉)
Department of General and Transplant Surgery, University Hospital Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany
e-mail: peter.schemmer@med.uni-heidelberg.de

2013; Menon et al. 2013; Schnitzbauer et al. 2010). Taken together, modern chemotherapy concepts can extend median survival in the palliative situation of HCC patients for up to 10.7 months (Rimassa and Santoro 2009).

Angiogenesis is also considered to be one of the hallmarks of cancer progression; when tumor growth exceeds a certain size, diffusion is no longer sufficient and the tumor cells express Hypoxia-inducible factor 1α (HIF-1α), which leads to angiogenesis via an upregulation of vascular endothelial growth factor (VEGF-A) (Hanahan and Weinberg 2000; Shweiki et al. 1992). Serum levels of VEGF-A are related to vascular invasion of HCC and linked to the central role of VEGF in signaling HCC progression (Mukozu et al. 2013).

Glycine, a non-essential amino acid, is non-toxic in dosages that have been used in clinical trials (Evins et al. 2000; Luntz et al. 2005). In previous studies, the inhibition of tumor growth by 5 % using dietary glycine was reported in rodent models of induced liver tumors in rats and subcutaneous melanoma models in mice (Rose et al. 1999a, b). Dietary glycine treatment has resulted in decreased breast adenocarcinoma growth in vivo (Amin et al. 2003). This has been linked to antiangiogenic effects; the proliferation of endothelial cells was decreased after glycine in vitro and tumors in glycine-fed animals showed significantly lower microvessel density (Amin et al. 2003; Rose et al. 1999b). In VEGF-stimulated endothelial cells, an inhibitory effect of glycine on the proliferation and migration of these cells was identified and linked to a function of the glycine receptor (GlyR), which is localized in the endothelial cell membrane (Yamashina et al. 2001, 2007). While glycine inhibits endothelial cell proliferation, there also seems to be a protective anti-apoptotic effect on endothelial cells. This is supported by the fact that apoptosis caused by VEGF depletion can be prevented by glycine (Zhang et al. 2000).

The anti-inflammatory and hepatoprotective effects of glycine have been demonstrated in experimental models of different types of liver injury (Bruns et al. 2011; Mikalauskas et al. 2011; Thurman et al. 1998; Zhong et al. 2003). In this context, Kupffer cells play a major role and are inhibited via the activation of the cells' GlyR (Wheeler et al. 1999). Direct cytoprotective effects on hepatocytes have also been reported (Bhattacharyya et al. 2012). There is indirect evidence for the presence of a GlyR in hepatocytes; glycine inhibits prostaglandin E2-mediated Ca^{2+} increase by boosting intracellular chloride levels (Qu et al. 2002). This effect seems to be particular to glycine and can be blunted by the presence of the GlyR antagonist, strychnine (Qu et al. 2002).

VEGF-A is a secreted homodimeric key pro-angiogenic protein with an approximate molecular weight of 45 kDa (Leung et al. 1989). At least six different splice variants of the protein are known to exist (Robinson and Stringer 2001). In endothelial cells, VEGF-A binds to the tyrosine-kinase receptors, VEGFR-1 and VEGFR-2 (Robinson and Stringer 2001; Neufeld et al. 1999). In highly vascularized malignancies, VEGF-A plays a pivotal role in vascularization, growth, and progression of HCC; monoclonal anti-VEGF antibodies can inhibit angiogenesis and have been shown to ultimately retard tumor growth in nude mice models (Smith et al. 2008; Kim et al. 1993). The endothelial response to VEGF-A leads to increased proliferation and migration of endothelial cells, vessel formation, and enhanced vascular permeability (Zhang et al. 2012). There is a strong positive correlation between VEGF-A expression and vascularization of HCC (Brodsky et al. 2007). VEGF-A levels in tumor tissue of HCC are usually elevated when compared to those in surrounding noncancerous liver tissues or cirrhotic livers without HCC (Brodsky et al. 2007; Yamaguchi et al. 1998; Iavarone et al. 2007).

The available treatment options for HCC are promising but far from being optimal (Camma et al. 2013; Rimassa and Santoro 2009). Moreover, the high costs of the present palliative treatment options are, especially in low income countries, not available to all persons in need (Kew 2012). Glycine, on the other hand, is inexpensive and easily available, thus making it worthwhile to investigate the amino acid's value in the treatment of HCC. The aim of this study was to evaluate any possible GlyR-dependent, VEGF-A-mediated, antiangiogenic effects on HCC cells in vitro.

Materials and methods

Cell culture

HepG2 and Huh7 cells were cultured in glycine-free DMEM with stable glutamine (Applichem, Darmstadt, Germany), supplemented with 10 % FBS (Biochrom AG, Berlin, Germany) and penicillin 100 U/ml/streptomycin 100 µg/ml (Biochrom AG, Berlin, Germany) at 37 °C in 5 % CO_2 humidified atmosphere. Cells were grown to 90 % confluence, the culture media was changed, and glycine (Sigma Aldrich, Saint Louis, USA) was added to achieve final concentrations of 0 (controls), 0.01, 0.1, 1, 2, 5 and 10 mM of the amino acid. In treatment experiments, cells were either cultured under normoxic or hypoxic conditions to stimulate HIF-1α-dependent, VEGF-A expression. In hypoxia experiments, cells were cultured using a modular incubator chamber (Billups-Rothenberg, Del Mar, USA) filled with 1 % O_2, 5 % CO_2, and 94 % N_2 (Rießner gase GmbH, Lichtenfels, Germany). In additional controls, the GlyR antagonist, strychnine (50 and 150 µM), was combined with 10 mM glycine.

Cell viability assay

After 48 h of cell culture incubation with and without glycine, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Carl Roth, Karlsruhe, Germany) was dissolved in PBS (PAA Laboratories GmbH, Pasching, Austria), added to the culture, and incubated for an additional 4 h at 37 °C. The supernatant was discarded and the plates were dried for 30 min at 37 °C. The precipitated formazan crystals were dissolved using 2-propanol (VWR, Darmstadt, Germany) and absorbance at 570 nm wavelength was measured using a spectrophotometer (Anthos Microsysteme, Krefeld, Germany).

Real-time quantitative polymerase chain reaction (RT-PCR)

After 24 h of cell culture incubation, mRNA was isolated using the RNeasy Mini Kit (Qiagen Gmbh, Hilden, Germany) and transcribed into cDNA using the First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Custom-made primers for VEGF-A [5′-CTTGCCTT GCTGCTCTACC-3′ (forward) and 5′-CACACAGGATGG CTTGAAG-3′ (reverse)] were used with GAPDH [5′-AGG GCTGCTTTTAACTCTGGT-3′ (forward) and 5′-CCC CACTTGATTTTGGAGGGA-3′ (reverse)] as the endogenous control. Quantitative real-time PCR was performed using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in triplicate and the relative gene quantification of the three independent experiments was analyzed using the ΔΔCT method using StepOne™ Software 2.1 (Applied Biosystems, Foster City, USA).

Western Blotting

After 24 h of cell culture incubation, cell lysates were prepared in RIPA buffer (Sigma Aldrich, Saint Louis, USA) using a proteinase inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). NuPAGE 4–12 % Bis–Tris Gel (Novex, Carlsbad, USA) electrophoresis of 20 µg of each protein sample was performed using XCell SureLock Mini-Cell module (Invitrogen, Carlsbad, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Munich, Germany) using XCell IITM Blot Module (Invitrogen, Carlsbad, USA). Membranes were blocked in PBS +0.1 % Tween with 5 % BSA (Serva Electrophoresis, Heidelberg, Germany) and incubated with primary antibodies at 4 °C for 12 h, followed by incubation with secondary antibodies for 1 h at room temperature. Films were developed using chemiluminescent substrate (Thermo Scientific, Rockford, USA) in a FUSION SL image acquisition system (Vilber Lourmat, Eberdharzell, Germany). Restore™ Western blot stripping buffer (Thermo Scientific, Rockford, USA) was used where appropriate. Antibodies against VEGF-A, Glycine receptors α1+α2 subunits (Abcamm, Cambridge, UK; ab46154 and ab23809), and β-actin (Sigma Aldrich, Saint Louis, USA; A1978) were used as primary antibodies and goat anti-rabbit and goat anti-mouse (sc-2004 and sc-2005; Santa Cruz Biotechnology Inc., Santa Cruz, USA) horseradish peroxidase-conjugated antibodies were used as secondary antibodies. Densitometric quantification was performed using ImageJ 14.3u (NIH, Bethesda, USA).

Statistics

For the analysis of parametric data, analysis of variance (ANOVA) was performed and for the analysis of non-parametric data, Kruskal–Wallis test followed by Dunn's post hoc test has been used where appropriate. A value of $p < 0.05$ was considered significant. All data are expressed as mean ± SD.

Results

Cell viability

The viability of cells was unchanged after an observation period of 48 h in all groups (data not shown).

RT-PCR

After 24 h of cell culture incubation, mRNA was isolated and transcribed to cDNA, before RT-PCR was performed to test for VEGF-A expression. In HepG2 cells, treatment with 1 and 10 mM of glycine significantly downregulated VEGF-A mRNA expression to 60 ± 8 and 58 ± 6 %, respectively (Fig. 1a), while in Huh7 cells, 10 mM of glycine significantly decreased VEGF-A mRNA expression to 65 ± 9 % (Fig. 1b) compared to the corresponding controls without glycine. No significant difference was found between 1 and 10 mM in HeG2 cells.

Western Blotting

In HepG2 and Huh7 cells, VEGF-A expression was decreased on protein level after glycine treatment. Under normoxic conditions, the administration of 1 and 10 mM of glycine significantly decreased the relative density of VEGF-A to 64 ± 10 and 63 ± 7 %, respectively (Fig. 2a). In Huh7 cells, 10 mM glycine significantly diminished VEGF-A levels to 69 ± 12 % (Fig. 2b) in comparison to

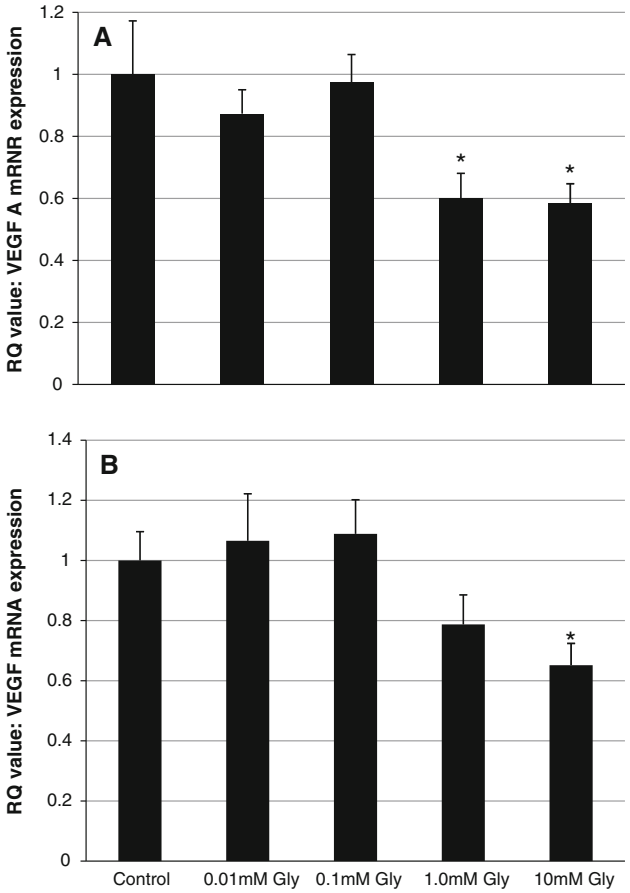

Fig. 1 VEGF-A expression (mRNA). Cells were treated using 0 (controls), 0.01, 0.1 1.0, and 10.0 mM glycine. **a** HepG2 cells; **b** Huh7 cells. $*p < 0.05$; *Gly* glycine

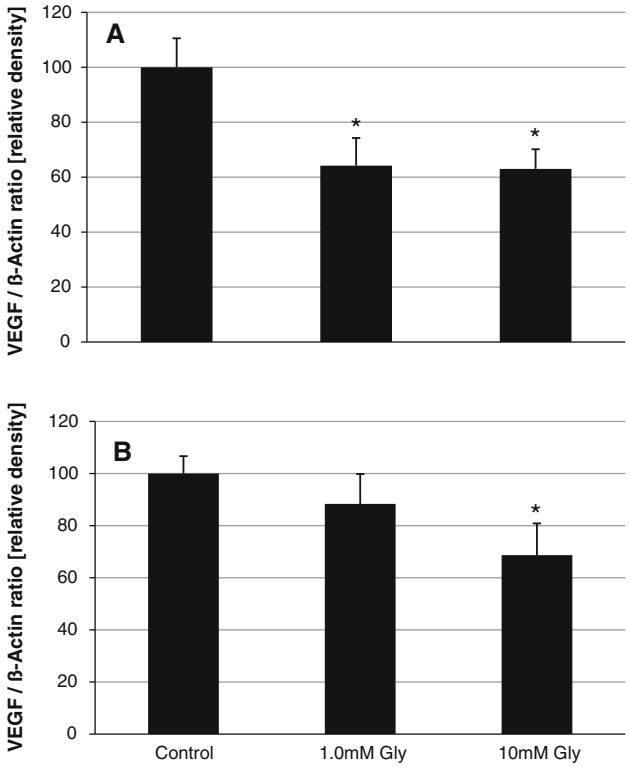

Fig. 2 VEGF-A expression (protein). Cells were treated using 0 (controls), 1.0, and 10.0 mM glycine. **a** HepG2 cells; **b** Huh7 cells. $*p < 0.05$; *Gly* glycine

the corresponding normoxic controls without glycine. The same effect was also observed under hypoxic conditions. The relative density of VEGF-A was notably reduced to 77 ± 13 and 74 ± 6 % when the cell cultures contained 1 and 10 mM of glycine, respectively (Fig. 3a) in comparison to the corresponding hypoxic controls without glycine. The most notable decline was observed in the 10 mM glycine experiment (Fig. 3b). This effect was completely blunted in both cell lines by the presence of 50 and 150 μM of the GlyR-specific inhibitor, strychnine (Fig. 3a, b). Evidence indicating the presence of GlyR was observed in whole cell lysates of both cell lines. Proteins of 48 kDa target molecular weight, representing the α1 and α2 subunits of the receptor, were expressed in both cell lines.

Discussion

Inhibition of angiogenesis is a promising strategy in modern chemotherapy especially in the case of highly vascularized tumors. This pathway is not only pivotal for neoplasms, but also involved in various stages of regeneration, embryology, and organ development (Dulmovits and Herman 2012). Angiogenesis regulation is highly redundant; when one pathway is downregulated, others become upregulated, leading to a "tumor escape" phenomenon and ultimately, to the failure of most antiangiogenic therapies (Bottsford-Miller et al. 2012).

Angiogenesis and vascularization are considered to belong to the essential mechanistic strategies of tumor progression and modern antiangiogenic substances such as the monoclonal VEGF antibody, Bevacizumab, can extend patient survival (Hanahan and Weinberg 2000; Giantonio et al. 2007; Hurwitz et al. 2004). In HCC, conventional chemotherapy strategies do not effectively extend overall survival (Cao et al. 2012; Wei et al. 2013). The introduction of Sorafenib, a multikinase inhibitor with antiangiogenic and antiproliferative properties, has been shown to extend median survival of patients with advanced HCC from 7.9 to 10.7 months (Rimassa and Santoro 2009).

Glycine is a simple, non-essential amino acid with normal blood levels between 200 and 400 μmol/L and can be easily administered orally or intravenously (Wheeler et al. 1999; Zhong et al. 2003; Luntz et al. 2005; Collentine 1948). The concentration of glycine used in the present study is significantly higher than what can normally be found in humans and achieved in clinical studies. The normal concentration of

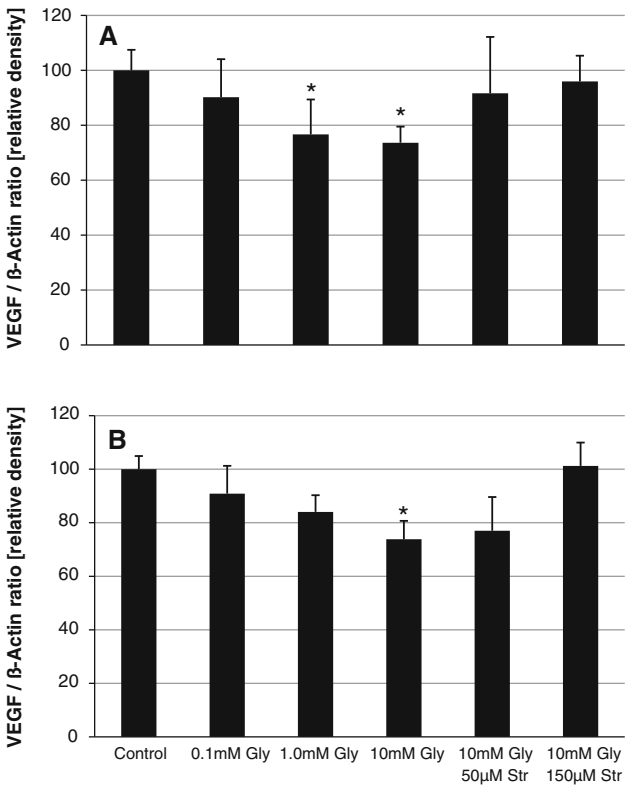

Fig. 3 VEGF-A expression (protein). Cells were treated under hypoxic conditions using 0 (controls), 1.0, and 10.0 mM glycine or 50 and 150 μM strychnine and 10 mM glycine (additional controls). **a** HepG2 cells; **b** Huh7 cells. *$p < 0.05$; *Gly* glycine, *Str* strychnine

glycine in blood plasma is 180–230 μM (Neeman et al. 2005). The extrapolation of concentrations that are effective in vitro to in vivo models is a non-trivial problem (Gulden and Seibert 2003; Halle 2003), but there is some data on this issue concerning glycine. In in vitro experiments using glycine, a media concentration of 10 mM is not uncommon and has been used to inhibit endothelial cell growth, while in in vivo models a standardized diet containing 5 % glycine is usually effective (Bruns et al. 2011; Ikejima et al. 1996; Mikalauskas et al. 2011; Rose et al. 1999b). The glycine plasma levels that can be achieved in rat models using a 5 % glycine diet usually reach approximately 1 mM (Ikejima et al. 1996). In clinical trials, oral daily dosages of up to 90 g have been applied without serious side effects and increased blood levels to more than 900 μM (Evins et al. 2000; Heresco-Levy et al. 1999).

In animal studies, glycine exhibited antiangiogenic effects against melanoma (Rose et al. 1999b). Moreover, glycine inhibits HCC induction in toxic models in rats (Rose et al. 1999a). In non-malignant liver tissue, glycine can ameliorate chemotherapy-induced liver damage via Kupffer cell-dependent mechanisms (Mikalauskas et al. 2011).

In the current study, glycine decreased VEGF-A in both the mRNA (Fig. 1) and protein levels that were expressed under normoxic (Fig. 2) and hypoxic (Fig. 3) conditions. There is indirect evidence that this mechanism is mediated via GlyR, since the effect was completely blunted by the presence of the GlyR-specific antagonist, strychnine (Fig. 3). In Western Blotting, the α1 and α2 subunits of GlyR were identified, which are essential for forming the ligand binding site of GlyR. Thus, the downregulation of VEGF-A by glycine can, at least in part, be explained by activation of the GlyR in cell lines. GlyR activation leads to an influx of cellular Cl^-, which subsequently reduces the concentration of cytosolic Ca^{2+} (Rajendra et al. 1997). This important intracellular messenger has been linked to activation of various components of the AP-1 transcriptional complex, which in turn regulates VEGF-A (Thiel et al. 2012; Salnikow et al. 2002). There could be possible effects that are mediated via the *N*-methyl-D-aspartate-receptor (NMDAR), which also has co-binding site for glycine (Johnson and Ascher 1987). There is evidence that NMDAR-dependent signaling plays a significant role in cancer growth (Abdul and Hoosein 2005; Choi et al. 2004; Liu et al. 2007). Recently, Yamaguchi et al. have detected the presence of a functional NMDAR in HepG2 and Huh7 cells (Yamaguchi et al. 2013). In their experiments, inhibition of NMDAR using MK-801, a specific inhibitor of the NMDAR-dependent Ca2+ channel not related to the glutamate and glycine binding sites and thus fully blocking NMDAR signaling, induced G1 cell cycle arrest and decreased cell proliferation. In contrast to these findings, no effect of glycine on cell proliferation was present in our study. The NMDARs glycine co-binding site can be activated by its specific agonist D-serin but is strychnine insensitive (Johnson and Ascher 1987; Kleckner and Dingledine 1988; Miyakawa et al. 2002). There are little to no D-serin-dependent effects on the GlyR (Curtis et al. 1968; Kuhse et al. 1995; Lynch 2004; Mori et al. 2002). Using strychnine as a specific antagonist for GlyR but not for the NMDAR glycine co-binding site, the glycine-dependent effects on VEGF signaling were completely reverted in our study. In conclusion, the effects observed in the present study can be attributed to the GlyR and seem not to be related to the glycine co-binding site of the NMDAR.

The current study has also demonstrated that the inhibitory effect of glycine on VEGF-A expression was present under hypoxic (Fig. 3) and normoxic (Fig. 2) conditions, thus giving further evidence that GlyR-dependent Ca^{2+} signaling is involved in both HIF-1α-dependent and -independent VEGF-A regulatory pathways. Interestingly, there is evidence that glycine might also blunt the endothelial response to VEGF-A via endothelial GlyR (Yamashina et al. 2001, 2007).

Besides the outcomes demonstrated here, glycine might have additional beneficial effects in conventional chemotherapy options. Effective chemotherapeutical

regimens for the treatment of colorectal metastases such as FOLFOX and FOLFIRI have known hepatotoxic side effects that can be ameliorated by the administration of glycine in animal experiments (Mikalauskas et al. 2011). While it is tempting to initiate clinical trials and simply add glycine in targeted and standard chemotherapy strategies for highly vascularized tumors like HCC, harmful effects have to be initially ruled out using in vivo tumor models. In fast proliferating human cancer cell lines, glycine has been identified as a potentially pro-tumorigenic agent (Jain et al. 2012). Inhibition of glycine metabolism has been shown to result in decreased proliferation rates of cancer cells (Jain et al. 2012). In the in vitro experiments in this study, neither direct toxic nor proliferative effects of glycine on HepG2 and Huh7 cells were seen.

The experiments carried out in this study can only serve as a model. Chemotherapy affects more than just one cell type, so the entire process of tumor progression and the topic of chemotherapy-induced liver injury are oversimplified in in vitro models. It is possible that glycine might affect HCC progression in multiple ways, since this amino acid can modulate immune responses, and thus, might modify widespread and local response to HCCs (Wheeler et al. 1999; Zhong et al. 2003). It remains to be verified whether or not the antiangiogenic effects of the amino acid identified in our study far outweighs any possible tumor-protective effects in vivo before wide-scale clinical trials can be justified.

Conclusion

Glycine is a potent, indirect inhibitor of in vitro tumor angiogenesis via the modulation of VEGF-A expression under normoxic and hypoxic cell culture conditions. This effect is mediated by GlyR and can, at least in part, be attributed to Ca^{2+}-related modulation of hypoxia-dependent and -independent transcription factors.

Acknowledgments The authors would like to thank Nadya Phillips-Houben for language editing.

Conflict of interest The authors of this manuscript have no conflicts of interest to disclose.

References

Abdul M, Hoosein N (2005) N-methyl-D-aspartate receptor in human prostate cancer. J Membr Biol 205(3):125–128. doi:10.1007/s00232-005-0777-0

Amin K, Li J, Chao WR, Dewhirst MW, Haroon ZA (2003) Dietary glycine inhibits angiogenesis during wound healing and tumor growth. Cancer Biol Ther 2(2):173–178

Bhattacharyya S, Ghosh J, Sil PC (2012) Iron induces hepatocytes death via MAPK activation and mitochondria-dependent apoptotic pathway: beneficial role of glycine. Free Radical Res 46(10):1296–1307. doi:10.3109/10715762.2012.712690

Bishayee A, Darvesh AS (2012) Angiogenesis in hepatocellular carcinoma: a potential target for chemoprevention and therapy. Curr Cancer Drug Targets 12(9):1095–1118

Bottsford-Miller JN, Coleman RL, Sood AK (2012) Resistance and escape from antiangiogenesis therapy: clinical implications and future strategies. J Clin Oncol Off J Am Soc Clin Oncol 30(32):4026–4034. doi:10.1200/JCO.2012.41.9242

Brodsky SV, Mendelev N, Melamed M, Ramaswamy G (2007) Vascular density and VEGF expression in hepatic lesions. J Gastrointest Liver Dis JGLD 16(4):373–377

Bruns H, Watanpour I, Gebhard MM, Flechtenmacher C, Galli U, Schulze-Bergkamen H, Zorn M, Buchler MW, Schemmer P (2011) Glycine and taurine equally prevent fatty livers from Kupffer cell-dependent injury: an in vivo microscopy study. Microcirculation 18(3):205–213. doi:10.1111/j.1549-8719.2010.00078.x

Buitrago-Molina LE, Vogel A (2012) mTor as a potential target for the prevention and treatment of hepatocellular carcinoma. Curr Cancer Drug Targets 12(9):1045–1061

Camma C, Cabibbo G, Petta S, Enea M, Iavarone M, Grieco A, Gasbarrini A, Villa E, Zavaglia C, Bruno R, Colombo M, Craxi A, group WEFs, group Ss (2013) Cost-effectiveness of sorafenib treatment in field practice for patients with hepatocellular carcinoma. Hepatology 57(3):1046–1054. doi:10.1002/hep.26221

Cao H, Phan H, Yang LX (2012) Improved chemotherapy for hepatocellular carcinoma. Anticancer Res 32(4):1379–1386

Choi SW, Park SY, Hong SP, Pai H, Choi JY, Kim SG (2004) The expression of NMDA receptor 1 is associated with clinicopathological parameters and prognosis in the oral squamous cell carcinoma. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol 33(9):533–537. doi:10.1111/j.1600-0714.2004.00226.x

Collentine GE Jr (1948) On the efficacy and safety of glycine administered by vein. J Lab Clin Med 33(12):1555–1562

Curtis DR, Hosli L, Johnston GA (1968) A pharmacological study of the depression of spinal neurones by glycine and related amino acids. Exp Brain Res 6(1):1–18

de Lope CR, Tremosini S, Forner A, Reig M, Bruix J (2012) Management of HCC. J Hepatol 56(Suppl 1):S75–S87. doi:10.1016/S0168-8278(12)60009-9

Di Marco V, De Vita F, Koskinas J, Semela D, Toniutto P, Verslype C (2013) Sorafenib: from literature to clinical practice. Annals Oncol Off J Eur Soc Med Oncol ESMO 24(Suppl 2):ii30–ii37. doi:10.1093/annonc/mdt055

Dulmovits BM, Herman IM (2012) Microvascular remodeling and wound healing: a role for pericytes. Int J Biochem Cell Biol 44(11):1800–1812. doi:10.1016/j.biocel.2012.06.031

El-Serag HB, Rudolph KL (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132(7):2557–2576. doi:10.1053/j.gastro.2007.04.061

Evins AE, Fitzgerald SM, Wine L, Rosselli R, Goff DC (2000) Placebo-controlled trial of glycine added to clozapine in schizophrenia. Am J Psychiatry 157(5):826–828

Giantonio BJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, Schwartz MA, Benson AB 3rd, Eastern Cooperative Oncology Group Study E (2007) Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J Clin Oncol Off j Am Soc Clin Oncol 25(12):1539–1544. doi:10.1200/JCO.2006.09.6305

Gulden M, Seibert H (2003) In vitro-in vivo extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations in vitro. Toxicology 189(3):211–222

Halle W (2003) The Registry of Cytotoxicity: toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. Altern Lab Animals ATLA 31(2):89–198

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100(1):57–70

Heresco-Levy U, Javitt DC, Ermilov M, Mordel C, Silipo G, Lichtenstein M (1999) Efficacy of high-dose glycine in the treatment of enduring negative symptoms of schizophrenia. Arch Gen Psychiatry 56(1):29–36

Hoffmann K, Schmidt J (2009) New surgical approaches in the treatment of hepatocellular carcinoma. Z Gastroenterol 47(1):61–67. doi:10.1055/s-0028-1109097

Hoffmann K, Glimm H, Radeleff B, Richter G, Heining C, Schenkel I, Zahlten-Hinguranage A, Schirrmacher P, Schmidt J, Buchler MW, Jaeger D, von Kalle C, Schemmer P (2008) Prospective, randomized, double-blind, multi-center, Phase III clinical study on transarterial chemoembolization (TACE) combined with Sorafenib versus TACE plus placebo in patients with hepatocellular cancer before liver transplantation—HeiLivCa [ISRCTN24081794]. BMC cancer 8:349. doi:10.1186/1471-2407-8-349

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350(23):2335–2342. doi:10.1056/NEJMoa032691

Iavarone M, Lampertico P, Iannuzzi F, Manenti E, Donato MF, Arosio E, Bertolini F, Primignani M, Sangiovanni A, Colombo M (2007) Increased expression of vascular endothelial growth factor in small hepatocellular carcinoma. J Viral Hepatitis 14(2):133–139. doi:10.1111/j.1365-2893.2006.00782.x

Ikejima K, Iimuro Y, Forman DT, Thurman RG (1996) A diet containing glycine improves survival in endotoxin shock in the rat. Am J Physiol 271(1 Pt 1):G97–G103

Jain M, Nilsson R, Sharma S, Madhusudhan N, Kitami T, Souza AL, Kafri R, Kirschner MW, Clish CB, Mootha VK (2012) Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. Science 336(6084):1040–1044. doi:10.1126/science.1218595

Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325(6104):529–531. doi:10.1038/325529a0

Kelley RK, Venook AP (2013) Novel therapeutics in hepatocellular carcinoma. Am Soc Clin Oncol Educ Book/ASCO Am Soc Clin Oncol Meeting 2013:137–142. doi:E10.1200/EdBook_AM.2013.33.e137

Kew MC (2012) Hepatocellular carcinoma in developing countries: prevention, diagnosis and treatment. World J Hepatol 4(3):99–104. doi:10.4254/wjh.v4.i3.99

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 362(6423):841–844. doi:10.1038/362841a0

Kleckner NW, Dingledine R (1988) Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. Science 241(4867):835–837

Kuhse J, Betz H, Kirsch J (1995) The inhibitory glycine receptor: architecture, synaptic localization and molecular pathology of a postsynaptic ion-channel complex. Curr Opin Neurobiol 5(3):318–323

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246(4935):1306–1309

Liu JW, Kim MS, Nagpal J, Yamashita K, Poeta L, Chang X, Lee J, Park HL, Jeronimo C, Westra WH, Mori M, Moon C, Trink B, Sidransky D (2007) Quantitative hypermethylation of NMDAR2B in human gastric cancer. Int J Cancer J Int cancer 121(9):1994–2000. doi:10.1002/ijc.22934

Lubienski A, Bitsch RG, Schemmer P, Grenacher L, Dux M, Kauffmann GW (2004) Long-term results of interventional treatment of large unresectable hepatocellular carcinoma (HCC): significant survival benefit from combined transcatheter arterial chemoembolization (TACE) and percutaneous ethanol injection (PEI) compared to TACE monotherapy. RoFo Fortschritte auf dem Gebiete der Rontgenstrahlen und der Nuklearmedizin 176(12):1794–1802. doi:10.1055/s-2004-813669

Luntz SP, Unnebrink K, Seibert-Grafe M, Bunzendahl H, Kraus TW, Buchler MW, Klar E, Schemmer P (2005) HEGPOL: randomized, placebo controlled, multicenter, double-blind clinical trial to investigate hepatoprotective effects of glycine in the postoperative phase of liver transplantation [ISRCTN69350312]. BMC Surgery 5:18. doi:10.1186/1471-2482-5-18

Lynch JW (2004) Molecular structure and function of the glycine receptor chloride channel. Physiol Rev 84(4):1051–1095. doi:10.1152/physrev.00042.2003

Mazzaferro V, Bhoori S, Sposito C, Bongini M, Langer M, Miceli R, Mariani L (2011) Milan criteria in liver transplantation for hepatocellular carcinoma: an evidence-based analysis of 15 years of experience. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc 17(Suppl 2):S44–S57. doi:10.1002/lt.22365

Menon KV, Hakeem AR, Heaton ND (2013) Meta-analysis: recurrence and survival following the use of sirolimus in liver transplantation for hepatocellular carcinoma. Aliment Pharmacol Ther 37(4):411–419. doi:10.1111/apt.12185

Mikalauskas S, Mikalauskiene L, Bruns H, Nickkholgh A, Hoffmann K, Longerich T, Strupas K, Buchler MW, Schemmer P (2011) Dietary glycine protects from chemotherapy-induced hepatotoxicity. Amino Acids 40(4):1139–1150. doi:10.1007/s00726-010-0737-6

Miyakawa N, Uchino S, Yamashita T, Okada H, Nakamura T, Kaminogawa S, Miyamoto Y, Hisatsune T (2002) A glycine receptor antagonist, strychnine, blocked NMDA receptor activation in the neonatal mouse neocortex. NeuroReport 13(13):1667–1673

Mori M, Gahwiler BH, Gerber U (2002) Beta-alanine and taurine as endogenous agonists at glycine receptors in rat hippocampus in vitro. J Physiol 539(Pt 1):191–200

Mukozu T, Nagai H, Matsui D, Kanekawa T, Sumino Y (2013) Serum VEGF as a tumor marker in patients with HCV-related liver cirrhosis and hepatocellular carcinoma. Anticancer Res 33(3):1013–1021

Neeman G, Blanaru M, Bloch B, Kremer I, Ermilov M, Javitt DC, Heresco-Levy U (2005) Relation of plasma glycine, serine, and homocysteine levels to schizophrenia symptoms and medication type. Am J Psychiatry 162(9):1738–1740. doi:10.1176/appi.ajp.162.9.1738

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z (1999) Vascular endothelial growth factor (VEGF) and its receptors. FASEB J Off Publ Fed Am Soc Exp Biol 13(1):9–22

Qu W, Ikejima K, Zhong Z, Waalkes MP, Thurman RG (2002) Glycine blocks the increase in intracellular free Ca2+ due to vasoactive mediators in hepatic parenchymal cells. Am J Physiol Gastrointest Liver Physiol 283(6):G1249–G1256. doi:10.1152/ajpgi.00197.2002

Rajendra S, Lynch JW, Schofield PR (1997) The glycine receptor. Pharmacol Ther 73(2):121–146

Rimassa L, Santoro A (2009) Sorafenib therapy in advanced hepatocellular carcinoma: the SHARP trial. Expert Rev Anticancer Ther 9(6):739–745. doi:10.1586/era.09.41

Robinson CJ, Stringer SE (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 114(Pt 5):853–865

Rose ML, Cattley RC, Dunn C, Wong V, Li X, Thurman RG (1999a) Dietary glycine prevents the development of liver tumors caused by the peroxisome proliferator WY-14,643. Carcinogenesis 20(11):2075–2081

Rose ML, Madren J, Bunzendahl H, Thurman RG (1999b) Dietary glycine inhibits the growth of B16 melanoma tumors in mice. Carcinogenesis 20(5):793–798

Salnikow K, Kluz T, Costa M, Piquemal D, Demidenko ZN, Xie K, Blagosklonny MV (2002) The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxia-inducible factor 1 in response to hypoxia. Mol Cell Biol 22(6):1734–1741

Sauer P, Kraus TW, Schemmer P, Mehrabi A, Stremmel W, Buechler MW, Encke J (2005) Liver transplantation for hepatocellular carcinoma: is there evidence for expanding the selection criteria? Transplantation 80(1 Suppl):S105–S108

Schnitzbauer AA, Zuelke C, Graeb C, Rochon J, Bilbao I, Burra P, de Jong KP, Duvoux C, Kneteman NM, Adam R, Bechstein WO, Becker T, Beckebaum S, Chazouilleres O, Cillo U, Colledan M, Fandrich F, Gugenheim J, Hauss JP, Heise M, Hidalgo E, Jamieson N, Konigsrainer A, Lamby PE, Lerut JP, Makisalo H, Margreiter R, Mazzaferro V, Mutzbauer I, Otto G, Pageaux GP, Pinna AD, Pirenne J, Rizell M, Rossi G, Rostaing L, Roy A, Turrion VS, Schmidt J, Troisi RI, van Hoek B, Valente U, Wolf P, Wolters H, Mirza DF, Scholz T, Steininger R, Soderdahl G, Strasser SI, Jauch KW, Neuhaus P, Schlitt HJ, Geissler EK (2010) A prospective randomised, open-labeled, trial comparing sirolimus-containing versus mTOR-inhibitor-free immunosuppression in patients undergoing liver transplantation for hepatocellular carcinoma. BMC Cancer 10:190. doi:10.1186/1471-2407-10-190

Shiah HS, Chen CY, Dai CY, Hsiao CF, Lin YJ, Su WC, Chang JY, Whang-Peng J, Lin PW, Huang JD, Chen LT (2013) Randomised clinical trial: comparison of two everolimus dosing schedules in patients with advanced hepatocellular carcinoma. Aliment Pharmacol Ther 37(1):62–73. doi:10.1111/apt.12132

Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359(6398):843–845. doi:10.1038/359843a0

Smith V, Wirth GJ, Fiebig HH, Burger AM (2008) Tissue microarrays of human tumor xenografts: characterization of proteins involved in migration and angiogenesis for applications in the development of targeted anticancer agents. Cancer Genomics Proteomics 5(5):263–273

Thiel G, Lesch A, Keim A (2012) Transcriptional response to calcium-sensing receptor stimulation. Endocrinology 153(10): 4716–4728. doi:10.1210/en.2012-1343

Thurman RG, Schemmer P, Zhong Z, Bunzendahl H, von Frankenberg M, Lemasters JJ (1998) Kupffer cell-dependent reperfusion injury in liver transplantation: new clinically relevant use of glycine. Langenbecks Archiv fur Chirurgie Supplement Kongressband Deutsche Gesellschaft fur Chirurgie Kongress 115:185–190

Wei Z, Doria C, Liu Y (2013) Targeted therapies in the treatment of advanced hepatocellular carcinoma. Clin Med Insights Oncol 7:87–102. doi:10.4137/CMO.S7633

Wheeler MD, Ikejema K, Enomoto N, Stacklewitz RF, Seabra V, Zhong Z, Yin M, Schemmer P, Rose ML, Rusyn I, Bradford B, Thurman RG (1999) Glycine: a new anti-inflammatory immunonutrient. Cell Mol Life Sci CMLS 56(9–10):843–856

Yamaguchi R, Yano H, Iemura A, Ogasawara S, Haramaki M, Kojiro M (1998) Expression of vascular endothelial growth factor in human hepatocellular carcinoma. Hepatology 28(1):68–77. doi:10.1002/hep.510280111

Yamaguchi F, Hirata Y, Akram H, Kamitori K, Dong Y, Sui L, Tokuda M (2013) FOXO/TXNIP pathway is involved in the suppression of hepatocellular carcinoma growth by glutamate antagonist MK-801. BMC Cancer 13:468. doi:10.1186/1471-2407-13-468

Yamashina S, Konno A, Wheeler MD, Rusyn I, Rusyn EV, Cox AD, Thurman RG (2001) Endothelial cells contain a glycine-gated chloride channel. Nutr Cancer 40(2):197–204. doi:10.1207/S15327914NC402_17

Yamashina S, Ikejima K, Rusyn I, Sato N (2007) Glycine as a potent anti-angiogenic nutrient for tumor growth. J Gastroenterol Hepatol 22(Suppl 1):S62–S64. doi:10.1111/j.1440-1746.2006.04655.x

Zhang Y, Ikejima K, Honda H, Kitamura T, Takei Y, Sato N (2000) Glycine prevents apoptosis of rat sinusoidal endothelial cells caused by deprivation of vascular endothelial growth factor. Hepatology 32(3):542–546. doi:10.1053/jhep.2000.16605

Zhang L, Wang JN, Tang JM, Kong X, Yang JY, Zheng F, Guo LY, Huang YZ, Zhang L, Tian L, Cao SF, Tuo CH, Guo HL, Chen SY (2012) VEGF is essential for the growth and migration of human hepatocellular carcinoma cells. Mol Biol Rep 39(5):5085–5093. doi:10.1007/s11033-011-1304-2

Zhong Z, Wheeler MD, Li X, Froh M, Schemmer P, Yin M, Bunzendaul H, Bradford B, Lemasters JJ (2003) L-Glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent. Current Opinion Clin Nutr Metab Care 6(2):229–240. doi:10.1097/01.mco.0000058609.19236.a4

Zhou Q, Wong CH, Lau CP, Hui CW, Lui VW, Chan SL, Yeo W (2013) Enhanced Antitumor Activity with Combining Effect of mTOR Inhibition and Microtubule Stabilization in Hepatocellular Carcinoma. Int J Hepatol 2013:103830. doi:10.1155/2013/103830