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Inhibition of Rho-Associated Kinase 1/2 Attenuates Tumor Growth in Murine Gastric Cancer[1,2]

Isabel Hinsenkamp*, Sandra Schulz†,3, Mareike Roscher‡,3, Anne-Maria Suhr‡, Björn Meyer†, Bogdan Munteanu†, Jens Fuchser§, Stefan O. Schoenberg‡, Matthias P.A. Ebert*, Björn Wängler‡, Carsten Hopf† and Elke Burgermeister*

*Dept. of Internal Medicine II, Universitätsmedizin Mannheim, Medical Faculty Mannheim, University Heidelberg, Mannheim, Germany; †Center for Applied Research in Biomedical Mass Spectrometry (ABIMAS) and Institute of Medical Technology of Heidelberg University and Mannheim University of Applied Sciences, Mannheim, Germany; ‡Dept. of Clinical Radiology and Nuclear Medicine (Molecular Imaging and Radiochemistry), Universitätsmedizin Mannheim, Medical Faculty Mannheim, University Heidelberg, Mannheim, Germany; §Bruker Daltonik GmbH, Bremen, Germany

Abstract

Gastric cancer (GC) remains a malignant disease with high mortality. Patients are frequently diagnosed in advanced stages where survival prognosis is poor. Thus, there is high medical need to find novel drug targets and treatment strategies. Recently, the comprehensive molecular characterization of GC subtypes revealed mutations in the small GTPase RHOA as a hallmark of diffuse-type GC. RHOA activates RHO-associated protein kinases (ROCK1/2) which regulate cell contractility, migration and growth and thus may play a role in cancer. However, therapeutic benefit of RHO-pathway inhibition in GC has not been shown so far. The ROCK1/2 inhibitor 1-(5-isoquinoline sulfonyl)-homopiperazine (HA-1077, fasudil) is approved for cerebrovascular bleeding in patients. We therefore investigated whether fasudil (i.p., 10 mg/kg per day, 4 times per week, 4 weeks) inhibits tumor growth in a preclinical model of GC. Fasudil evoked cell death in human GC cells and reduced the tumor size in the stomach of CEA424-SV40 TAg transgenic mice. Small animal PET/CT confirmed preclinical efficacy. Mass spectrometry imaging identified a translatable biomarker for mouse GC and suggested rapid but incomplete *in situ* distribution of the drug to gastric tumor tissue. RHOA expression was increased in the neoplastic murine stomach compared with normal non-malignant gastric tissue, and fasudil reduced (auto) phosphorylation of ROCK2 at THR249 *in vivo* and in human GC cells *in vitro*. In sum, our data suggest that RHO-pathway inhibition may constitute a novel strategy for treatment of GC and that enhanced distribution of future ROCK inhibitors into tumor tissue may further improve efficacy.

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Abbreviations: GC, gastric cancer; CEA, carcinoembryonic antigen; FASU, fasudil; FTICR, fourier transform ion cyclotron resonance; MALDI-MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging; MAPK, mitogen-activated protein kinase; MLC, myosin light chain; PET/CT, positron emission/computer tomography; ROCK, Rho-associated kinase; SV40, simian virus 40; TAg, large T-Antigen; WB, Western blot; WT, wild-type

Address all correspondence to: Elke Burgermeister, Ph.D., Dept. of Medicine II, Universitätsklinikum Mannheim, Universität Heidelberg, Theodor-Kutzer Ufer 1-3, D-68167, Mannheim, Germany.
E-mail: elke.burgermeister@medma.uni-heidelberg.de

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Introduction

Gastric cancer (GC) is the third leading cause of cancer-related death world-wide [1,2]. Patients frequently present with advanced stage GC where curative treatment is not possible and the 5-year overall survival rate is less than 30%. Thus, novel treatment options need to be developed. Comprehensive genomic approaches have recently classified molecular subtypes of GC into at least four different genetic profiles [3–5]. In this context, three seminal papers corroborated the small GTPase RHOA as a major oncogenic driver mutation for the diffuse-type GC in humans (Kakiuchi et al., 2014 [5]; Wang et al., 2014 [4]; TCGA, 2014 [3]). The RHOA-signaling pathway is activated by integrins, growth factor, cytokine and G-protein-coupled receptors in response to signals from extracellular matrix and adhesion molecules, hormones and nutrients [6]. Active GTP-bound RHOA binds to a wide series of downstream effectors (>20), for example the RHO-associated protein kinases (ROCK1/2), and leads to an allosteric activation of the serine/threonine kinase domain and to autophosphorylation [7–10]. The ROCK1 and 2 enzymes are partially redundant, although tissue specific distribution has been reported. They phosphorylate an array of substrate proteins including cytoskeleton components such as myosin light chain (MLC) that promote cell contractility, adhesion and migration in normal and malignant cells. Thereby, ROCKs regulate cellular functions such as growth, invasion and cell viability and are involved in hypertension, Alzheimer's disease and cancer [11–13]. Hence, ROCK1/2 are attractive drug targets for disease prevention or therapy. Potent and selective ROCK1/2 inhibitors are under current (pre)clinical development, with fasudil [1-(5-isoquinoline sulfonyl)-homopiperazine] (HA-1077) approved for the clinical treatment of cerebral vasospasms during subarachnoid hemorrhage in China and Japan [14]. The compound has been shown to exert anti-tumor efficacy in rodent xenografts including breast, myeloma, lung, melanoma, glioblastoma and head-and-neck cancer [15–18].

RHO and ROCK isoforms are expressed in human GC cells [19,20], and RHOA expression and/or mutations have been associated with poor clinical prognosis in patients with diffuse GC [21–23]. Inhibition of RHOA/ROCK signaling promotes apoptosis and reduces proliferation of human GC cells [24–26]. We therefore hypothesized that pharmacological inhibition of ROCK1/2 by fasudil may exert therapeutic benefit *in vivo,* in mice with spontaneous genetically driven gastric carcinoma as a preclinical model of human GC. The transgenic C57BL/6 J mouse strain CEA424-SV40 TAg expresses the viral oncogene "large T-antigen" (TAg) from the Simian Virus 40 (SV40) under the control of the promoter of the human carcinoembryonic antigen (CEA) specifically in the lower part of the stomach (pylorus) and develops highly proliferative intraepithelial gastric carcinomas within 2 months of age and with 100% penetrance [27]. We show here both *in situ* drug distribution and metabolism together with preclinical efficacy of fasudil on tumor growth *in vivo* in murine GC and *in vitro* in human GC cell lines. In sum, our data propose that inhibition of the "oncogenic driver" RHO signaling pathway by marketed ROCK1/2 inhibitors may constitute a future novel therapy of human GC that could be further improved by next generation drugs with enhanced tumor penetration.

Materials and Methods

Animals

Transgenic CEA424-SV40 TAg C57BL/6 J mice with gastric carcinoma were described elsewhere [27,28]. Animal studies were conducted in agreement with ethical guidelines of the University of Heidelberg and approved by the government authorities (Az 35–9185.82/G-176/12).

Reagents

Acetonitrile (ACN), trifluoroacetic acid (TFA) and general chemicals were from Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany). Fasudil was from Selleckchem (Houston, TX). Antibodies were phosphorylated (P) P-ROCK2 (T249, #ab83514, Abcam, Cambridge, UK), Ki67 (#550609), unphosphorylated/general (G) G-ROCK2 (#610624) (both from BD Biosciences, Heidelberg, Germany), RHOA (STA-403-A-CB, Biocat, Heidelberg, Germany), P-MLC2 (#3671), P-ERK1/2(p44/p42) (#4370), G-ERK1/2(p44/p42) (#9102), P-P38 (#4511), G-P38 (#9218) (all from Cell Signaling), HSP90 (sc-7947, Santa Cruz Biot., CA). MALDI peptide calibration standard II (#222570), 2,5-dihydroxybenzoic acid (DHB, #209813) and indium tin oxide (ITO) slides were from Bruker Daltonik (Bremen, Germany), Isopentane (GPR RECTAPUR) from VWR (Darmstadt, Germany), FSC22 Frozen Section Compound from Leica Biosystems (Wetzlar, Germany) and Tissue-Tek Cryomolds from Sakura Finetek (Heppenheim, Germany). [^{18}F]-FDG was purchased from ZAG Zyklotron AG (Karlsruhe, Germany).

Cell Culture and Assays

Human embryonic kidney cells transformed with SV40 large T-Antigen (HEK293T) and GC cell lines (AGS, MKN45) (all from the American Type Culture Collection, Rockville, MD) were maintained as described previously [29]. Cell viability was measured by 1-(4,5-dimethylthiazol-2-yl) 3,5-diphenyl-formazan (MTT) assay (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer.

Protein Extraction, GTPase Pull-Down, Immunoprecipitation (IP) and Western Blot (WB)

All methodologies were performed as described before [30]. RHOA GTPase pull-down assay was performed as recommended by the manufacturer (STA-403-A-CB, Biocat, Heidelberg, Germany).

Immunohistochemistry (IHC) and Immunofluorescence (IF) Microscopy

Hematoxylin-eosin (H&E) and antibody stainings were performed as described [29,30]. For IHC, 3,3′-diaminobenzidine was used as a substrate (brown color). IF stainings were analyzed using a fluorescence microscope (Axiovert 200 M, Carl Zeiss MicroImaging GmbH, Germany) and software (Axiovision, release 4.4). Manual counting of signals ($n > 50$ per field, $n = 5$ fields per image) was done with Image J (imagej.nih.gov/ij).

Reverse Transcription PCR (RT-PCR) and Quantitative PCR (qPCR)

Primers are listed in Table S1. All methods were conducted as published [30,31].

MALDI-MS Imaging

Male C57BL/6 J and CEA424-SV40 TAg transgenic mice (C57BL6 background) (3 months of age, average body weight 20 g) received food and water *ad libitum* until 3 h before drug administration. Fasudil monohydrochloride [1-(5-isoquinolinesulfonyl)-homopiperazine] (HA-1077) was dissolved in sterile PBS and administered i.p. as a single dose of 30 mg/kg. Animals were treated for 10, 30, or 60 min