Inhibition of experimental HCC growth in mice by use of the kinase inhibitor DMAT

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Abstract. The multi-kinase-inhibitor Sorafenib has been shown to prolong survival of patients suffering from hepatocellular carcinoma (HCC). We investigated effects of the serine/threonine kinase inhibitor 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazol (DMAT) on experimental HCC growth, and identified mechanisms and target kinases of DMAT. Our results show that DMAT application in vivo reduced tumor growth in a xenotransplant model by interference with tumor cell proliferation. Biochemical parameters and histology following DMAT administration revealed no alterations in liver tissue. Similar to Sorafenib, DMAT interfered with NFκB activation and Wnt-signaling. Of the kinases inhibited by DMAT at almost equimolar IC50, CK2 and PIM-3 were found to be over-expressed or more active in hepatoma cells and human HCC tissue. Knockdown of PIM-3 or CK2 by shRNA revealed that both kinases are important for hepatoma cell proliferation and survival. In conclusion, DMAT reduces HCC growth by interference with NFκB- and Wnt-signaling. PIM-3 and CK2 seem to be important target kinases. Inhibition of these kinases by application of inhibitors, e.g., DMAT, might represent a promising therapeutic approach in future HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is among the most prevalent malignant tumors worldwide (1). Overall prognosis is poor and curative treatment by resection or liver transplantation is not possible in the majority of patients. In advanced stages, limited palliative treatment options are available, which are limited due to impaired metabolic function of the underlying cirrhotic livers of patients (2). Chemotherapeutic agents widely employed in other gastrointestinal cancers, e.g., doxorubicin, cisplatin, or 5-fluorouracil (5-FU) (3), have not been found to significantly prolong survival in HCC (4). Recently, the multi-kinase-inhibitor Sorafenib has been shown to exert anti-tumor activity and to prolong patient survival (5,6). Sorafenib, which acts as a tyrosine kinase inhibitor, targets VEGFR2 and 3, PDGFR, Flt-3, c-Kit and Raf (7,8).

In 2004, 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) was described as a novel CK2-inhibitor (9,10), blocking protein kinase CK2 over 1300-fold more efficiently than CK1 (11). CK2 is a ubiquitously expressed serine/threonine-kinase, composed of two catalytic α- or α'-subunits and two regulatory β-subunits. It catalyzes phosphorylation of proteins involved in cell cycle regulation, cellular survival and differentiation (12). Although expression of CK2 mainly occurs in embryonic tissues, it has also been detected in fast proliferating tissues of adult organisms, including colorectal, breast, pulmonary and kidney cancers (13). It has been shown that DMAT is also able to inhibit serine/threonine kinases PIM-1, PIM-3 and PKD1 at almost equimolar IC50 compared to CK2 (14).

We investigated the effect of kinase inhibition by DMAT in a mouse model of experimental HCC (xenografted HepG2 cells in NMRI mice) as well as in vitro. Our results show profound anti-tumor effects which seem to be dependent on NFκB inhibition and inhibition of Wnt-signaling. Knockdown experiments of DMAT target kinases revealed that effects occurred possibly due to inhibition of CK2 and PIM-3.
Materials and methods

Animal models of hepatocellular carcinoma. HepG2 (5.0x10⁶) human hepatoma cells were suspended in sterile physiologic NaCl solution and injected subcutaneously into the flank of 6-8-week old male NMRI mice (Harlan Winkelmann GmbH, Germany). Animals were kept in a light- and temperature-controlled environment and provided with food and water ad libitum. Tumor size was determined by using a caliper square. When subcutaneous tumors reached a diameter of 6-7 mm, daily i.p. treatment with DMAT (500 µg/kg in DMSO/PBS) or vehicle (DMSO/PBS) was started. After 10 days of treatment animals were sacrificed and samples were collected. Tumor and liver tissue samples were fixed in 4% phosphate-buffered formalin or snap-frozen in liquid nitrogen. Alkaline amino transferase (ALT) and aspartate amino transferase (AST) levels were determined using an automated procedure on a Cobas Mira (Roche, Mannheim, Germany). All mice received humane care according to the guidelines of the National Institute of Health as well as to the legal requirements in Germany. The study protocol complied with the Institute's guidelines and was approved by the Government of Lower Franconia (Würzburg, Germany, file number 54-2531.31-3/06) before the beginning of the experiments.

Human HCC samples. Human HCC tissues and non-neoplastic liver tissues were obtained from patients (Childs A/B cirrhosis) undergoing surgical resection. Tissue samples were snap-frozen and stored at -80°C. Informed consent was obtained from all patients, and the study was approved by the local Ethics Committee (Regensburg, Germany). CDNA of human hepatocytes was kindly provided by Dr M. Dandri, University Medical Center Hamburg-Eppendorf, Germany.

Dosage and application routes. The kinase inhibitor DMAT (2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazol, Merck Biosciences GmbH, Schwalbach am Taunus, Germany) was dissolved in DMSO at 50 mM and diluted to final concentrations in cell culture medium for in vitro experiments or in PBS for in vivo experiments. Control solutions containing the appropriate amount of DMSO in cell culture medium for in vitro experiments or in PBS for in vivo experiments were used. Concanavalin A (ConA) was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). 20 mg/kg were administered to mice intravenously in 200 µl pyrogen-free saline. Sorafenib (Bayer AG, Leverkusen, Germany) was kindly provided by Professor M. Ocker, University of Marburg, Germany.

Cell culture. The human hepatoma cell lines HepG2 and Huh-7 were maintained in DMEM medium, the mouse hepatoma cell line Hepa1-6 was maintained in RPMI medium (both: Invitrogen GmbH, Karlsruhe, Germany). Primary mouse hepatocytes were isolated and cultured as described previously (15). All media contained 10% fetal calf serum (FCS; Invitrogen GmbH) and 1% penicillin-steptomycin (Invitrogen GmbH). Primary mouse hepatocytes were isolated and cultured as described previously (15). All media contained 10% fetal calf serum (FCS; Invitrogen GmbH) and 1% penicillin-steptomycin (Invitrogen GmbH).

Analysis of cellular proliferation and viability. Cellular proliferation was measured using a cell counter (CASY TT, Roche, Basel, Switzerland). The percentage of dead cells was determined after 72 h of incubation with DMAT or vehicle. Cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) assay.

NFkB-activity assay and β-catenin-regulated transcription (CRT). HepG2 cells were transfected in 24-well plates with 0.4 µg of luciferase reporter pB2LUC, containing firefly luciferase under the control of 2 NFkB binding sites. CRT was monitored using a luciferase reporter system consisting of Super8 x TOPFlash and Super8 x FOPFlash. Plasmids were kindly provided by R.T. Moon (Howard Hughes Medical Institute, Seattle, WA). The TOPFlash plasmid (M50) contains 8 TCF/LEF binding sites enhancing firefly luciferase expression. The control plasmid FOPFlash (M51) carries mutant TCF/LEF binding sites. 24 h after transfection cells were assayed using a Reporter Assay Systems (Promega GmbH, Mannheim, Germany).

CK2-activity assay. Kinase-activity was measured as described previously (16).

Determination of caspase-3-activity. Caspase-3-activity was determined using a colorimetric Assay Kit (Sigma-Aldrich Chemie GmbH) according to the manufacturer's instructions.

Immunohistochemistry and H&E staining. Paraffin sections of 5 µm were cut and stored at room temperature until use. Routine histology (hematoxylin and eosin staining; H&E) was performed in order to evaluate basic histomorphological features of the specimens. TUNEL staining was performed on formalin-fixed and paraffin-embedded tissue sections using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and as described previously (17). Immunohistochemical staining procedures were applied as described before (18). Sections were deparaffinized using graded alcohols. After microwave antigen retrieval (10 mmol/l citrate buffer, pH 6.0; 8 min at 560 W and 8 min at 800 W), the sections were incubated overnight using an antibody specific for Ki-67 (mouse monoclonal anti-Ki-67; 1:500, Dako, Glostrup, Denmark). The primary mouse antibody was detected using the EnVision Detection System (Dako). Visualization was performed using diaminobenzidine (DAB) as the chromogen substrate (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Slides were counterstained with hematoxylin. The stained slides were digitalized using the ImageAccess Enterprise 9.0 software (Imagic Bildverarbeitung, Glattbrugg, Switzerland). The extensity of Ki-67 was measured by mean % of all cancer cells at 4 different high-power fields (magnification x400) per tumor specimen.

Real-time RT-PCR

Detection of mRNA by RT-PCR. To verify altered gene expression RNA was transcribed into cDNA using the Verso cDNA Kit (Thermo Fisher Scientific, Waltham, MA, USA). Oligonucleotides for subsequent PCR-reactions were obtained from Metabion International AG ( Martinsried, Germany) and are summarized in Table I. Real-time RT-PCR was performed using the CFX™ real-time system (Bio-Rad, Munich, Germany) and reagents..
from ABgene® (Epsom, UK). To confirm amplification specificity, PCR products were subjected to melting curve analysis and gel electrophoresis.

Preparation of cell lines stably expressing shRNA. ShRNA expression vectors were based on the lentiviral pLKO.1 construct (RNAi Consortium vector collection and purchased from Sigma-Aldrich Chemie GmbH. Target sequences for shRNA directed against PIM-3: 5'-CGC CTG TCA GAA GAT GAA CAT-3'; directed against CK2ap: 5'-CAT GGA GTC CTG TGG CAT CCA C-3'; directed against E. coli polymerase (shNeg; kindly provided by R. Everett, Glasgow; target sequence: 5'-TTA TCG CGC ATA TCA CGG GGG-3') was used as a negative control. Transduced cells were selected with puromycin (2 µg/ml).

Statistical analysis. The results were analyzed using Student's t-test if two groups were compared and the Dunnett's test if more groups were tested against a control group. If variances were heterogeneous in the Student's t-test, the results were analyzed using the Welsh test. The data are expressed as the mean ± SEM. P≤0.05 was considered significant.

Results

DMAT reduces hepatoma cell proliferation and growth of experimental HCC in mice without affecting the liver. To investigate effects of the serine/threonine kinase inhibitor DMAT on HCC growth we first measured proliferation and induction of apoptosis in vitro in two human hepatoma cell lines, HepG2 and Huh-7. We found that after 72 h of incubation proliferation was significantly reduced by 1 µM DMAT in HepG2 cells and by 10 µM in Huh-7 cells (Fig. 1A and B), while caspase-3-activity was not induced (Fig. 1C). We also measured caspase-3 activation at 24 h of incubation, since caspase-3 activation would precede the loss of viability. Here we were able to detect apoptosis induction at concentrations >30 µM in HepG2 and by 10 µM in Huh-7 cells (Fig. 1A and B), indicating that DMAT primarily interferes with cell proliferation, but also has the potential to induce apoptosis. In vivo, we induced experimental HCC by subcutaneous injection of HepG2 human hepatoma cells into NMRI nude mice and treated mice with DMAT starting when tumors reached a diameter of 6-7 mm. After 10 days of treatment, blood, tumors and livers of mice were harvested. Our results show that
DMAT interferes with NFκB-and Wnt-signaling, but not with angiogenesis. To elucidate pathways affected by DMAT, we measured NFκB-activity, Wnt-signaling, and markers for angiogenesis in hepatoma cells. Using luciferase reporter assays we found that DMAT interfered with NFκB-activity (Fig. 4A) and CRT, as a marker for activity of the tumor relevant Wnt-signaling pathway (Fig. 4C). These results are corresponding to those obtained using the multi-kinase inhibitor Sorafenib, which also reduced NFκB-activity (Fig. 4B) and wnt-signaling (Fig. 4D). Expression of conductin, another marker for Wnt-signaling activity, was also found to be significantly reduced by DMAT incubation in vitro (Fig. 4E), as well as in vivo in tumors of DMAT-treated mice (Fig. 4F). Angiogenesis in these tumors did not seem to be affected by DMAT treatment, since VEGF expression was not altered, as detected by real-time RT-PCR (Fig. 4G) and plasma ELISA (Fig. 4H). We did not observe alterations in angiopoietin 1 or 2 expressions in experimental HCC following DMAT treatments (data not shown).

Activity and expression of DMAT target kinases in hepatoma cells and HCC. Although DMAT has been developed as a specific CK2 inhibitor, it has recently been shown to inhibit several other kinases. Among those, PIM-1, PIM-3 and PKD1 show a similar IC₅₀ compared to CK2 (14). We investigated expression levels of these kinases as well as expressions of the catalytic CK2 subunits alpha (α) and alpha prime (αp) in primary mouse hepatocytes (PH) in comparison to mouse hepatoma cells (Hepa1-6) and found both CK2 subunits as well as PIM-3 to be over-expressed (Fig. 5A). Real-time RT-PCR revealed that PIM-3 was also over-expressed in human hepatoma cells (Fig. 5B) and it has been reported that PIM-3 was found over-expressed in human HCC (19). Since CK2 subunits can be differentially combined to form active CK2 enzyme, we measured CK2-activity in human HCC and tumor surrounding liver tissue of 14 patients by in vitro kinase assay (ivKA), and detected significantly increased CK2-activity in HCC samples (Fig. 5C). We also detected increased kinase-activity for CK2 in intrahepatic or subcutaneous experimental HCC in mice (data not shown). Therefore, CK2 and/or PIM-3 might represent targets for DMAT in HCC.

Knockdown of CK2 subunits or PIM-3 interferes with hepatoma cell viability and proliferation. To determine the contribution of the DMAT target kinases CK2 or PIM-3 to hepatoma cell survival we established hepatoma cell lines stably expressing shRNA directed against CK2 or PIM-3 (Fig. 6A). shRNA against CK2 subunits or PIM-3 (Fig. 6A) while depletion of PIM-3 additionally induced apoptosis in hepatoma cells (Fig. 6C). Knockdown of CK2 subunits or PIM-3 interferes with hepatoma cell viability and proliferation.

Combination of DMAT and chemotherapy. Up to now, targeted therapy with Sorafenib is able to prolong survival of patients with HCC (5,6). Nevertheless it is likely that resistance towards therapy might occur, either by escape mutants of target kinases or activation of alternative pathways, e.g., GGF-driven, similar to other angiostatic agents (20). Therapeutic failure due to secondary resistance might be less frequent employing combinational therapy. Therefore, we investigated a combination

tumor growth was significantly slower in mice treated with DMAT (Fig. 2A). Measurement of the proliferation marker Ki67 by immunohistochemical staining in tumor tissue revealed significantly decreased proliferation (example in Fig. 2B, quantified in Fig. 2C). We also measured apoptosis induction in tumor tissues by TUNEL staining (Fig. 2D) and caspase-3-activity as a marker for apoptosis (C) were measured. *P≤0.05 for DMAT vs. solvent incubated cells.

Figure 1. DMAT interferes with proliferation of human hepatoma cell lines. The human hepatoma cell lines HepG2 (filled bars) and Huh7 (open bars) were incubated in the presence of increasing concentrations of DMAT for 72 h. Cell viability (A), cell proliferation (B), and caspase-3-activity as a marker for apoptosis (C) were measured. *Ps0.05 for DMAT vs. solvent incubated cells.
of DMAT with sorafenib, doxorubicin (Doxo) or 5-fluorouracil (5-FU) in vitro in HepG2 (Fig. 7A) and Huh-7 (Fig. 7B) human hepatoma cell lines. Measurement of cell viability revealed that, similar to observations in Fig. 1, both lines responded differently towards DMAT and chemotherapeutics. In general, DMAT was able to enhance effects of single treatment, though there were no enhancing effects in sorafenib treatment of Huh-7 cells.

Discussion

Targeted therapy with sorafenib has become the standard of care for patients with advanced HCC (BCLC C). Currently other agents, for example, sunitinib, brivanib, axitinib, gefitinib, lanitib, erlotinib, are under investigation in clinical trials in the first- and second-line settings, either as monotherapy or in combinations. Various cancer-driving targets have been identified e.g., VEGF, VEGF receptors, FGF, FGF receptors, EGF receptor, HGF receptor and mTOR (8). Up to now the only agent which has been approved to be capable of prolonging HCC patient survival is the multi-kinase inhibitor sorafenib (5,6), though several other substances are under investigation in clinical trials (8).

We found that the serine/threonine kinase inhibitor DMAT is able to reduce experimental HCC growth in mice. Detrimental effects on liver tissue, either healthy or regenerating, were not detected, although DMAT predominantly seems to interfere with proliferation. Recently it has been shown that DMAT reduces cell proliferation and interferes with Wnt-signaling in biliary tract cancer cell lines (21). We also detected interference with Wnt signaling in vitro as well as in vivo, showing that DMAT may be classified as Wnt pathway-inhibitor, although it also inhibits NFκB activation in hepatoma cells and therefore is not specific for the Wnt pathway. Both mechanisms of action

Figure 2. DMAT treatment interferes with tumor-growth in experimental HCC mice. Experimental HCC was induced in NMRI nude mice by subcutaneous injection of HepG2 cells. DMAT was applied to animals intraperitoneally at 500 µg/kg when tumors reached a diameter of 6-7 mm. Control mice received DMAT-vehicle diluted to a concentration corresponding to the DMAT group. Application was continued for 10 consecutive days. Subsequently, mice were sacrificed and tumor size was measured using a caliper (A). Proliferation in tumors described in (A) was measured by Ki67-staining (B), the amount of proliferating cells in tumors of 3 DMAT- and 3 vehicle-treated animals described in (A) was quantified (C). Induction of apoptosis by DMAT-treatment in these animals was measured by TUNEL-staining (D) and caspase-3-activity ELISA (E), n=7-11; *P≤0.05 for DMAT vs. DMAT-vehicle treated animals.
against hepatoma cell survival can be attributed to its originally
described mode of action as a CK2 inhibitor. Moreover, recent
findings reveal that CK2 induced phosphorylation might be a
prerequisite for ubiquitination and degradation of tumor
suppressor proteins e.g., PML (22). CK2 has also been shown
to contribute to activation of raf (23), topoisomerase II (24),
the p38 signal transduction pathway (25), and NfκB signaling
(26,27). CK2-transgenic mice more frequently developed
T-cell lymphoma, which was enhanced by over-expression of
c-myc or loss of p53 (28,29). Using site-specific CK2 transgenic
mice, it has been shown that over-expression of CK2α
promotes mammary gland tumorigenesis (30) by mechanisms involving
NFκB activation and activation of the Wnt-signaling pathway
(31). It has also been shown that CK2 over-expression is able
to interfere with apoptosis in prostate-and colon-carcinoma
cell lines (32,34), and that inhibition of CK2α expression by
antisense oligonucleotides induces apoptosis (35). Using DMAT
we detected apoptosis induction only at high concentrations
in vitro and there was no induction of apoptosis visible in
mice, either in tumor tissue or in liver tissue. Stable knockdown
of CK2 catalytic subunits also did not induce apoptosis in
hepatoma cells. In our hands, DMAT treatment did not interfere
with angiogenesis, though CK2 has been reported to suppress
neovascularization (36). These findings point to a DMAT target
besides CK2, which is crucially involved in HCC growth. In
fact it has been described that DMAT inhibits kinases other
than CK2 (11) and we identified PIM-3 as a major DMAT
target kinase involved in hepatoma cell proliferation and
apoptosis. PIM-3 was originally identified as a depolarization-
induced gene, KID-1, in PC12 cells, a rat pheochromocytoma
cell line (37). Subsequently, it has been demonstrated that PIM-3
gene transcription was enhanced in the EWS/ETS-induced
carcinoma cells (38), suggesting
the involvement of PIM-3 in tumorigenesis. In fact, PIM-3
expression was found to be enhanced in carcinomas but not in
normal tissues of human organs, including the liver (19),

Figure 3. Application of DMAT does not induce liver damage in mice. Plasma transaminases ALT and AST of animals described in Fig. 2 were measured at
the day of sacrifice (A). Integrity of liver histology was determined by H&E staining (B). As a parameter for metabolic liver function expression of albumin
was measured by real-time RT-PCR (C). Possible induction of apoptosis by DMAT-treatment was measured by caspase-3-activity ELISA (D) and TUNEL-
staining (E). DMAT effects on regenerating liver were measured by plasma transaminases ALT and AST 20 h after administration of concanavalin A (ConA)
to mice partially pretreated with DMAT (F).

A

B

C

D

E

F
PIM-3 transgenic mice developed DEN-induced HCC with a higher incidence and a heavier burden, implicating that PIM-3 accelerates HCC development (42).

Our in vitro and in vivo experiments indicate that the main effect of DMAT is interference with hepatoma cell viability and proliferation and we tried to elucidate mediators and signal transduction pathways. We found that DMAT in vitro as well as in vivo interfered with Wnt-signaling. It has been shown that activation of the Wnt-signaling pathway (43) is triggered by CK2-dependent phosphorylation of β-catenin at position T 393, which provokes its stabilisation and protection from degradation by the proteasome (44). Subsequently, target genes of the Wnt-signaling pathway are induced, e.g., conductin, which might be a regulator of intensity and duration of Wnt-signaling (45). It is also possible that conductin itself might be able to promote tumor growth (46). Moreover, using colorectal carcinoma cell lines it has been shown that conductin, by interaction with centrobes and the mitotic spindle, might induce chromosomal instability (47).

Another effect observed in our experiments was DMAT-induced inhibition of NFκB activity. Here, also CK2 might play a crucial role. CK2 has been found to phosphorylate IkBα at a number of sites, including serine 283, 289, 293 and threonine 291, triggering its degradation and thereby activation of NFκB (48). NFκB-activity in many cases is associated with the expression of cytoprotective proteins, which subsequently confer a survival advantage to tumor cells (49).

Figure 4. DMAT interferes with NFκB activation and wnt-signaling. HepG2 cells were transfected with a reporter construct expressing luciferase under the control of an NFκB-dependent promoter and incubated in the presence of 10 or 20 µM DMAT (A) or 1 or 10 µM Sorafenib for 24 h (B). *P<0.05 for DMAT- or sorafenib-treated vs. vehicle-treated cells. HepG2 cells (C-E) and tumors of animals described in Fig. 2 (F and G) as well as plasma of these animals (H) were further examined for mechanisms of tumor growth reduction. Activity of the wnt-signaling pathway was measured by a luciferase-reporter-assay for β-catenin-regulated transcription (CRT; C, DMAT; D, Sorafenib) using cell lysates containing equal amounts of protein. Conductin expression in HepG2 cells (E) and tumor tissue (F) was measured by real-time RT-PCR. VEGF-expression, as a marker for ongoing angiogenesis, was measured by real-time RT-PCR (G) and plasma ELISA (H). *P<0.05 for DMAT-or Sorafenib vs. vehicle treatment.
Though we found that PIM-3 is a crucial factor in hepatoma cell survival and protection from apoptosis there are so far no reports linking this kinase to Wnt-signaling or NFκB-activity. Moreover, own preliminary experiments did not reveal an inhibitory effect of a stable PIM-3 knockdown on Wnt-signaling or NFκB-activity (data not shown). Therefore, PIM-3 seems to support tumor relevant signaling pathways, which are currently under investigation.

Resistance towards at first hand successful tumor therapy might be prevented by a combination of substances targeting either different pathways and preventing tumor cell replication as much as possible or by targeting different key factors in an exceptionally important pathway, e.g., Wnt-signaling or angiogenesis. We investigated a combination of DMAT with sorafenib or the conventional chemotherapeutics 5-FU or doxorubicin and found that DMAT enhanced their effects on tumor cells. Effects were in a range of 10-20% within 72 h of incubation. Interestingly, DMAT also increased the effects of sorafenib, indicating differences in their spectra of target kinases. We also observed that hepatoma cell lines HepG2 and Huh-7 responded differently towards single or combinatory treatment. These observations support the discussion that therapy has to...
become more individualized and that due to their repertoire of over-activated tumor promoting proteins, not all patients might profit from standardized therapy to the same extent.

In conclusion, our observations indicate that DMAT, alone or in combination with chemotherapeutics, might become a well tolerated new tool which is worth further exploitation for treatment of HCC in clinical trials.

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