Synergistic anti-proliferative effect of metformin and sorafenib on growth of anaplastic thyroid cancer cells and their stem cells

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Abstract. Sorafenib, a multikinase inhibitor has recently been approved for the treatment of radio-iodine refractory thyroid carcinoma. However, toxic side effects may lead to dose reduction. In the present study, we analyzed whether a combined therapy with metformin may allow a dose reduction of sorafenib without loss of effectiveness at the same time. In HTh74 anaplastic thyroid carcinoma (ATC) cells and its derived doxorubicin-resistant HTh74Rdox cell line, the growth inhibitory effect of sorafenib with or without metformin was investigated. Furthermore, an analysis of cell cycle arrest in response to sorafenib was performed and the ability of a combined treatment to induce apoptosis was analyzed. In addition, the effects on clonal growth and formation of stem cell-derived spheres were assayed. The influence of sorafenib and metformin on MAP kinase pathway was investigated by analysis of ERK phosphorylation. Sorafenib and metformin synergistically inhibited growth of the two thyroid cancer cell lines, with a more pronounced effect on the doxorubicin-resistant HTh74Rdox cell line. The two drugs also synergistically decreased sphere formation, which suggested a specific effect on thyroid cancer stem cells. The addition of metformin enabled a 25% dose reduction of sorafenib without loss of its growth inhibitory efficacy. Sorafenib and metformin synergistically decreased the proliferation of ATC cell lines and the outgrowth of their derived cancer stem cells. A combined treatment enabled a significant dose reduction of sorafenib. In respect to frequent toxic side effects, clinical studies in future should demonstrate whether the addition of metformin may be an advantage in the chemotherapy of patients with radio-iodine-resistant thyroid cancer.

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Introduction

Anaplastic thyroid carcinoma (ATC), which accounts for 2-5% of thyroid carcinomas, is one of the most aggressive and resistant human malignancies (1,2). The disease is usually advanced by the time of diagnosis and 75% of the patients develop distant metastasis during later progression. The multimodal treatment of ATC includes surgical extirpation, radiotherapy and chemotherapy (usually doxorubicin or paclitaxel) (3). Despite combined therapy, the prognosis of the disease is poor with an average survival time of only 6-8 months (3,4).

Sorafenib, a multikinase inhibitor, targets kinases of different receptors such as VEGFR-2, VEGFR-3, PDGFR, RET and BRAF and thus exhibits antitumor and anti-angiogenic activities. Sorafenib has been approved for the treatment of advanced renal cell carcinoma, unresectable hepatocellular carcinoma and tested in preclinical and ongoing clinical studies in breast carcinoma, colon cancer and melanoma (5). Different studies in patients with undifferentiated and radioiodine refractory-differentiated thyroid carcinoma (DTC) have demonstrated superiority of sorafenib over the standard care with doxorubicin (6-9). Therefore, the FDA has recently approved this chemotherapeutic drug for treatment of differentiated thyroid cancer that does not respond to radio-iodine therapy (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm376443.htm).

However, patients treated with sorafenib may develop toxic side effects including hand-foot syndrome, rash, fatigue, diarrhea, and hypertension (10). Since these side-effects are, at least in part, dose-dependent, a reduction of sorafenib dose without reducing the therapeutic effect may be of high clinical value.

Metformin, a commonly prescribed, well-tolerated anti-diabetic agent, may be a candidate for such a combined therapy. Its anti-proliferative effect on thyroid carcinoma cells was clearly demonstrated (11). Moreover, metformin amplifies the anti-mitogenic effect of chemotherapeutic agents such as doxorubicin and cisplatin whose dose may be reduced in a combined therapy (11).

In the present study, a synergistic effect of sorafenib and metformin on the growth inhibition of anaplastic thyroid cancer was demonstrated. Sorafenib inhibited anaplastic

thyroid cell growth by blocking cell cycle progression and inducing apoptosis. On the molecular level, sorafenib decreased the cell growth rate by inhibiting MAPK signaling pathway. Furthermore, sorafenib inhibited clonal cell growth and thyroid cancer sphere formation, a characteristic of cancer stem cells. Of note, metformin amplified the anti-mitogenic effect of sorafenib and synergistically decreased clonal cell growth and sphere formation in ATC cells. In conclusion, the results showed that combined chemotherapy of metformin with sorafenib reduced the dose-dependent side-effects of this chemotherapeutic drug.

Materials and methods

Cell cultures. The HTh74 anaplastic thyroid cancer cell line was kindly provided by Dr Heldin, Uppsala, Sweden. Cells were cultured in F12 medium supplemented with 10% fetal calf serum (FCS, v/v), 1% MEM (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin.

The stable doxorubicin-resistant thyroid carcinoma cell line HTh74Rdox was established as described below (12). Briefly, the HTh74Rdox cell line was derived by continuous exposure of this cell line to 0.5 μg/ml doxorubicin for >6 months. The IC_{50} value for doxorubicin was 153.53±16.43 μg/ml, which corresponds to an 85-fold increase compared to doxorubicin-sensitive parental HTh74 cells. Flow cytometric analysis after Hoechst 33342 dye staining demonstrated that ~80% of the doxorubicin-resistant cells were detectable as a side population cell fraction, enriched with cancer stem cells that expressed transporters of the ATP-binding cassette (ABC) gene family (12).

Monolayer cultures of human thyrocytes isolated from nodular goiters of 6 patients undergoing thyroidectomy were established and cultured as described previously (13,14). This study was approved by the Local Ethics Committee. In all cases informed consent was obtained.

Cell viability assay. Cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described by the manufacturer (Sigma-Aldrich, St. Louis, MO, USA). All the experiments were repeated at least three times in quadruplicate.

Cell cycle and apoptotic analysis. Analysis of cell cycle arrest was performed in low-density cultures ($1x10^5$ cells/6 cm dish, 40-50% confluence), whereas apoptosis was investigated in high-density cultures ($4x10^5$ cells/6 cm dish, confluent >90%) (15). The cell cycle was analysed by measuring the amount of propidium iodide (PI) incorporation into cellular DNA in ethanol-fixed cells. Apoptotic and necrotic cell death was analyzed by double staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany). The green and red fluorescence of Annexin V/PI-stained viable cells and PI-stained fixed cells were analyzed with a FACSVerse flow cytometer (BD Biosciences) using a peak fluorescence gate to exclude cell aggregates during cell cycle analysis. The number of viable (Annexin V^-/PI^-), apoptotic (Annexin V^+/PI^-) and necrotic (Annexin V^+/PI^+) cells and the proportion of cells in different cell cycle phases were calculated with the FACSDiva software (BD Biosciences).

Colorimetric assay of caspase-3 activity. Caspase-3 activity ($2x10^6$ cells seeded in 10 cm dishes) was determined using a colorimetric assay kit (Sigma-Aldrich) according to the manufacturer's instructions. The intra- and inter-assay CV of caspase-3 assay was 4.49 and 6.46%, respectively.

In vitro clonal analysis. HTh74 and HTh74Rdox cells were plated at clonal density (200 cells/well) in triplicates in 6-well plates and treated with the indicated concentrations (1-10 μM) of sorafenib with or without metformin. Formed colonies were stained with Giemsa and the percentage of cells that initiated a clone was determined as cloning efficiency.

Sphere formation assay. Tumor spheres that consist of stem cells and their progenitor cells were generated by placing HTh74 cells ($1x10^4$ cells/ml) into serum-free DMEM/F12 medium containing B27 (1:50 dilution), bFGF (20 ng/ml) and EGF (20 ng/ml), as described previously (11). Sphere-forming efficiency (SFE) was calculated as the number of sphere-like structures (large diameter >50 μm) formed in 7 days divided by the original number of cells seeded and expressed as a percentage mean (± SD).

Western blot analysis. Cells following different treatments were lysed with RIPA buffer. The proteins were treated with 4X sample buffer containing dithiothreitol and boiled for 10 min. An equal amount of protein (30 μg) was subjected to 12.5% SDS polyacrylamide gel and separated proteins were transferred to NC membranes. The membranes were blocked in 5% skim milk for 1 h at room temperature. The immunoblots were incubated overnight at 4˚C with anti-cyclin D1, anti-ERK, and anti-phosphorylated ERK1/2 (Thr^{202} and Tyr^{204}) antibodies (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in 5% BSA/TBST at a dilution of 1:1,000, 1:1,0000, and 1:2,000, respectively. The following day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The immunoreactive bands were detected with a chemiluminescence substrate kit (ProteinSimple, Santa Clara, CA, USA) under the FluorChem FC2 system.

Flow cytometry for side population cells. To isolate the thyroid cancer side population fraction, FACS was performed using the Hoechst 33342 dye staining method as described previously (16). Briefly, cancer cells were labeled with 5 μg/ml Hoechst 33342 dye (Sigma-Aldrich) either alone or in combination with 50 μM verapamil (Sigma-Aldrich), which is an inhibitor of ABCG2 transporter. The cells were counterstained with 1 μg/ml PI to exclude dead cells. A 350-nm UV laser was used to excite Hoechst 33342 dye and PI. Analysis was performed on a fluorescence-activated cell sorter (BD Biosciences) using a dual-wavelength analysis (blue, 424-444 nm and red, 675 nm).

Statistical analysis. Statistical analysis was performed with SPSS13.0 software. Numerical data are expressed as mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

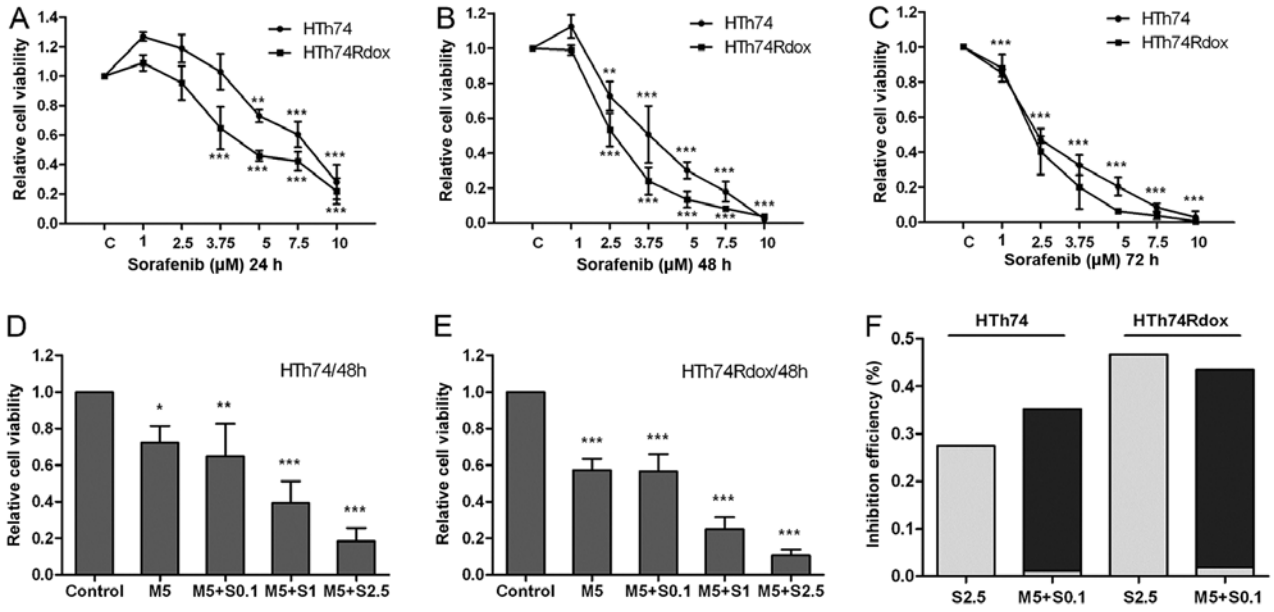

Figure 1. Sorafenib and metformin inhibit cancer cell viability. (A-C) HTh74 and HTh74Rdox cells were cultured with increasing doses of sorafenib (1-10 μM) for 24-72 h incubation. The percentage of surviving cells in relation to the controls was determined by MTT. (D-E) Cell viability of HTh74 and HTh74Rdox cells treated with increasing doses of sorafenib with metformin (5 μM) for 48 h measured by MTT. (F) Comparison of the growth inhibitory efficacy between sorafenib alone and sorafenib combined with metformin. Data are presented as the mean of at least three independent experiments in six replicates. (C), *P<0.05, **P<0.01, ***P<0.001 vs. control.

Results

Inhibition of cell growth by sorafenib with or without metformin. Human HTh74 and HTh74Rdox ATC cells were treated with various concentrations of sorafenib for 24-72 h. As shown in Fig. 1A-C, sorafeninb significantly inhibited growth of the HTh74 and HTh74Rdox cells in a dose- and time-dependent manner. The mean EC_{50} values in the 48-h cell viability assay were ~3.28 μM for HTh74 cells, and 2.47 μM for HTh74Rdox cells, indicating that HTh74Rdox cells, which are enriched with cancer stem cells, were more sensitive to sorafenib than their parental HTh74 cells.

To determine whether metformin influenced the anti-mitogenic effect of sorafenib, viability of HTh74 and HTh74Rdox cells was analysed after combined treatment with the two drugs. As shown in Fig. 1D-E, there was additional growth inhibition in the combination group. Metformin (5 μM) combined with 0.1 μM sorafenib exhibited the equivalent growth inhibitory effect as 2.5 μM as monotherapy with sorafenib in HTh74 and HTh74Rdox cells (~35-45% inhibition efficiency, Fig. 1D-F).

Induction of apoptosis by sorafenib and metformin. To investigate the mechanism of growth inhibition by sorafenib and to evaluate whether sorafenib and metformin act synergistically, the induction of apoptosis was analyzed after different treatments. As shown in Fig. 2, sorafenib and metformin as monotherapy enhanced apoptosis of HTh74 and HTh74Rdox cells. The addition of 5 μM sorafenib or 5 μM metformin increased the percentage of apoptotic cells from 1.7 to 4.1% and 3.1%, respectively, in HTh74 cells and from 1.7 to 4.3% and 4.9%, respectively, in HTh74Rdox cells (Fig. 2A and B). In the sorafenib/metformin group, the induction of apoptosis was much more pronounced (5.4% in HTh74 cells and 17.9% in HTh74Rdox cells) compared to sorafenib or metformin as monotherapy.

In addition, agent-induced apoptosis was assayed by detection of caspase-3 activity in HTh74 and HTh74Rdox cells. In response to sorafenib or metformin treatment, caspase-3 activity markedly increased by 18.4 and 52.0%, respectively, in HTh74 cells and 77.3 and 131.0%, respectively, in HTh74Rdox cells (Fig. 2C). Compared to the results of Annexin V-FITC and PI staining, the combination of sorafenib with metformin was more effective in inducing caspase-3 activity than as monotherapy (caspase-3 activity was increased by 60.2 and 222.2% in HTh74 and HTh74Rdox cells, respectively). Thus, in HTh74Rdox the pro-apoptotic effect of sorafenib and metformin was more pronounced than that in HTh74 cells.

Sorafenib and metformin cause cell cycle arrest. The effect of sorafenib and metformin on the cell cycle progression was analyzed by flow cytometry after 24-h treatment.

Sorafenib and metformin treatment led to the accumulation of cells in G1 phase with a consecutive decrease in the percentage of cells in S phase in HTh74 and HTh74Rdox cells (Fig. 3). The effect on cell cycle arrest induced by sorafenib was weaker than that by metformin. In the combination group, similar data were obtained although with a more pronounced decrease of cells in S phase than by sorafenib alone.

Inhibition of colony and tumor sphere formation by sorafenib. The self-renewal capacity of anaplastic thyroid cancer cells was analyzed by clonal formation and sphere formation assays. Colony formation assay was performed only in HTh74Rdox cells which are enriched with cancer stem cells and are more clonogenic than HTh74 cells (11,12). As observed in Fig. 4A, after treatment with 0.1 μM sorafenib

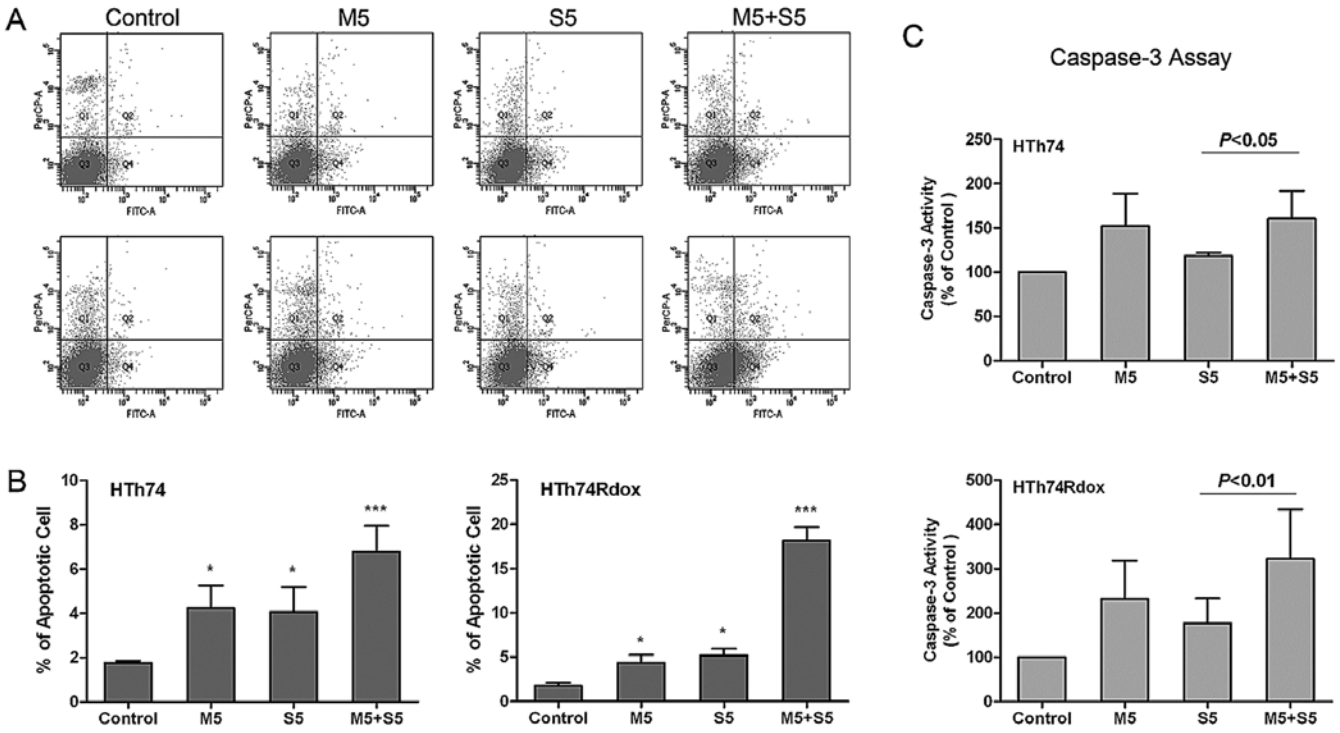

Figure 2. Induction of apoptosis by sorafenib and metformin. (A-B) Apoptosis of HTh74 (upper four dot plots) and HTh74Rdox (bottom 4 dot plots) cells in response to sorafenib (5 μM), metformin (5 μM) or a combination for 24 h was determined by Annexin V/PI staining. (B) The percentage of apoptotic cells are indicated from at least two independent experiments. (C) *P<0.05 and ***P<0.001 vs. control. (C) Relative caspase-3 activity of HTh74 and HTh74Rdox cells following treatment with sorafenib (5 μM), metformin (5 μM) or a combination for 24 h. Caspase-3 activity in untreated cells was set as 100%. Data are presented as the mean of three independent experiments in triplicate.

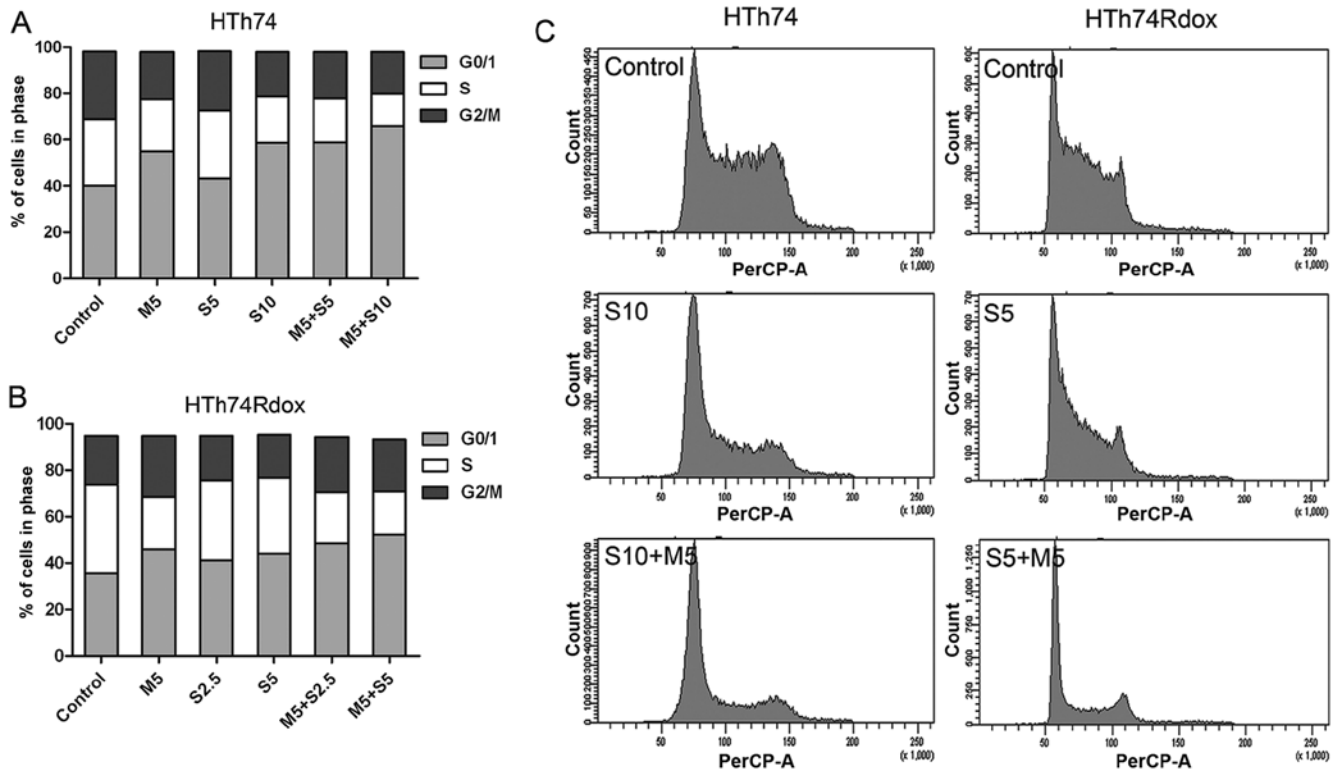

Figure 3. The combination of sorafenib and metformin blocked the cell cycle in G0/1 phase. HTh74 and HTh74Rdox cells were exposed to indicated concentrations of sorafenib, metformin, or a combination for 24 h. Cell cycle progression was analyzed by flow cytometry after propidium iodide (PI) staining.

or 2.5 μM metformin, the number of HTh74Rdox colonies formed was significantly reduced. Combination of 2.5 μM metformin with 0.01 or 0.1 μM sorafenib resulted in the further decrease of clonogenicity.

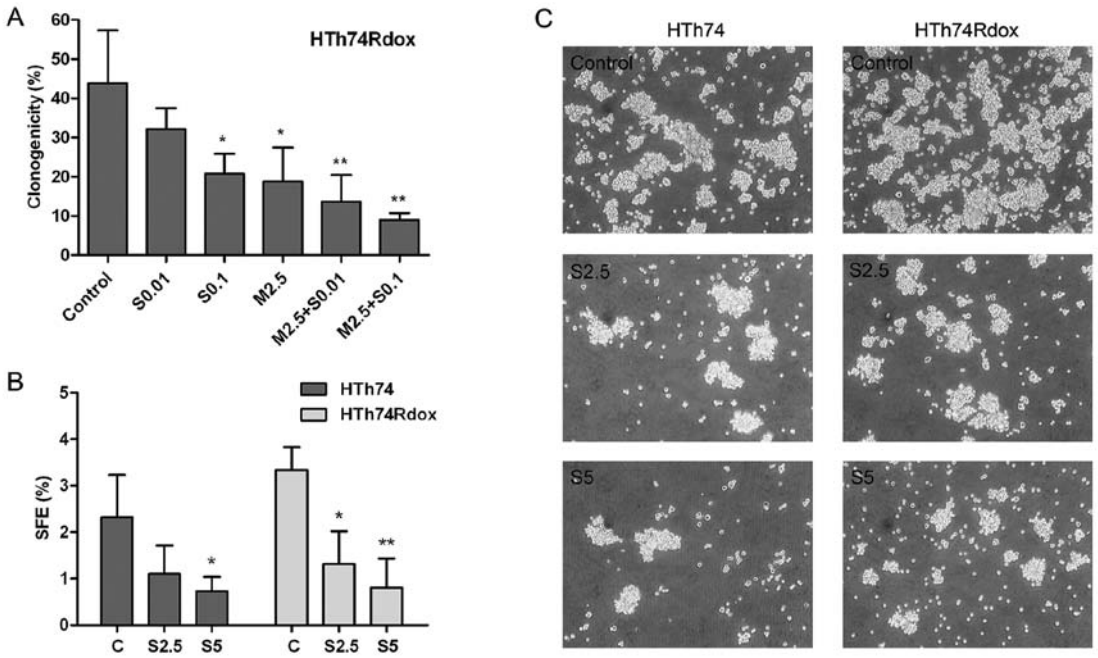

Figure 4. Colony formation and sphere formation efficiency following treatment with sorafenib in the presence or absence of metformin. (A) HTh74Rdox cells were grown in 6-well plates (200 cells/well). After 24 h, the culture medium was replaced with fresh medium containing sorafenib (0.01 and 0.1 μM), metformin (2.5 μM) or a combination or 2% fetal bovine serum as control. After 14 days, the colonies were stained with crystal violet and counted. (C) *P<0.05 and **P<0.01 vs. control. (B) Sphere formation efficiency (SFE) of HTh74 and HTh74Rdox cells was calculated as the number of spheres formed in 7 days divided by the original number of cells seeded and expressed as a percentage of means ± SD. Data shown are representative of three independent experiments. (C) *P<0.05 and **P<0.01 vs. control. (C) Representative spheres formed by HTh74 and HTh74Rdox cells growing in the sphere medium for 7 days with or without sorafenib.

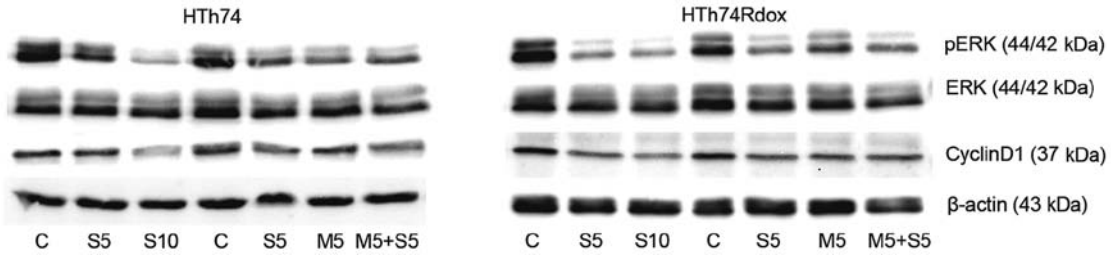

Figure 5. Sorafenib and metformin targeted MAPK pathway. Sorafenib and metfomin inhibited ERK phosphorylation. HTh74 and HTh74Rdox cells were treated with sorafenib in the presence or absence of metformin (5 μM) for 24 h. Cell lysates were then analyzed for ERK phosphorylation at Thr^{202} and Tyr^{204}.

The effect of sorafenib on HTh74 and HTh74Rdox cells, in the presence or absence of metformin, was investigated by sphere formation assay. In response to increasing doses of sorafenib, SFE was significantly lower in the two cell lines with ~52 and 61% reduction at the concentration of 2.5 μM sorafenib and 69 and 76% reduction at the concentration of 5 μM sorafenib in HTh74 and HTh74Rdox cells, respectively (Fig. 4B and C). In the presence of 5 μM metformin, the sphere formation efficiency was almost completely suppressed by 2.5 μM sorafenib (data not shown).

Sorafenib and metformin inhibit cell growth via MAPK pathway. To evaluate the effect of sorafenib, and sorafenib plus metformin on a pathway involved in growth inhibition and targeted by the two drugs, phosphorylation of ERK, a key protein of MAPK pathway, was analyzed in HTh74 and HTh74Rdox cells. Western blotting revealed that sorafenib markedly decreased the phosphorylation of ERK in a dose-dependent manner (Fig. 5A). Metformin also decreased the phosphorylation of ERK. However, a synergistically inhibitory effect of sorafenib plus metformin was not observed. The expression of cyclin D1 was reduced following sorafenib treatment with or without metformin.

Discussion

Removal of the tumor mass and subsequent ablation of the remaining thyroid cancer tissue by radioiodine therapy is a prerequisite for cure of thyroid cancer. Therefore, anaplastic thyroid carcinomas that lack radioiodine uptake have a very poor prognosis (17). Furthermore, life expectancy of patients with progressive local and metastatic well-differentiated thyroid cancer that secondarily became refractory to radioiodine treatment is also very limited (6).

As an evolving new strategy to improve prognosis of these thyroid carcinomas, multi-targeted tyrosine kinase inhibitors have been investigated in clinical studies (18). Recently, the FDA has approved sorafenib as the first drug of this group (http://

www.fda.gov). Sorafenib, an orally ingested drug, exerts its anti-angiogenic and anti-mitogenic effect by targeting BRAF, VEGFR1 and 2, RET and thereby important growth-regulating signaling pathways of thyroid cancer (19). In differentiated thyroid cancer the role of genetic aberrations in the RET-RAS-RAF-MAPK signaling pathway in tumor pathogenesis and progression is well established (20). Rearrangements in the RET proto-oncogene which is involved in the initiation of tumor formation are detectable in up to 25% of PTCs (21). Twenty nine to 69% of PTCs harbor a BRAF V600E mutation, which is associated with recurrent and persistent disease (21). RAS mutations and downstream signaling PIK3CA mutations occur in almost 50% of FTCs and >10% of Hurthle cell carcinomas (21).

In a xenograft model it was demonstrated that sorafenib obstructs RAF kinases and thereby inhibits growth of ATC cells (20). Furthermore, sorafenib inhibited the growth and angiogenesis of orthotopic ATC xenografts in nude mice (22). These carcinoma cells lacked any known molecular aberrations which argues against the hypothesis that the effect of sorafenib is limited to tumors with these mutations. Subsequent findings demonstrated that PTC cells carrying the RET/PTC1 rearrangement were more sensitive to sorafenib than those carrying a BRAF mutation (23).

Clinical studies confirmed the efficacy of sorafenib for patients with radioiodine refractory-differentiated thyroid carcinoma (DTC). A recent meta-analysis which included seven studies on radioiodine-refractory DTC, showed a partial response in 22% and stable disease in 52% of the patients (24). Median progression-free survival was 12.4 month (95% CI: 10.4-14.7). Sorafenib has also been utilized for patients with advanced ATC (9). Two out of 20 patients had a partial remission and 5 had stable disease with a duration of 4 months (range, 3-11 months). At least in patients with radioiodine-refractory thyroid carcinomas with a poor prognosis multi-kinase inhibitors such as sorafenib are considered promising.

However, toxic side effects such as hand-foot syndrome, diarrhea, fatigue, rash and weight loss that occur in up to 80% of sorafenib-treated patients are a concern (24). Due to the side effects dose reduction was necessary in >60% of the patients and in 6-25% of patients treatment was even discontinued (24). Therefore, adjuvant therapy that allows dose reduction of sorafenib without decreasing its efficacy may be an option to overcome this problem.

Metformin, a widely used, well-tolerated antidiabetic drug, has recently been demonstrated to potentiate the anti-mitogenic effects of doxorubicin and cisplatin in ATC cells (11). The combined therapy with metformin enabled a significant reduction of these chemotherapeutic drugs without reducing their anti-proliferative capacity (11).

In the present study, we have demonstrated that the anti-mitogenic effect of sorafenib was potentiated by the addition of metformin. The addition of metformin allowed a dose reduction of sorafenib by up to 25% without a decrease of the growth-inhibitory effect.

Sorafenib reduced viability of ATC cells by blocking cell cycle progression via G0/1 phase arrest and S phase inhibition and by inducing cell apoptosis. The effects were more pronounced when metformin was added to the culture.

Tumor sphere formation and clonal growth which reflect self-renewal and characteristic proliferation pattern, are a hallmark of cancer stem cells (12,25). In the present results, sorafenib significantly reduced clonal growth of HTh74Rdox cells. In addition, the drug inhibited tumor sphere formation by decreasing tumor sphere number and size. Again, metformin amplified the effect of sorafenib in the two experiments. These data suggest that sorafenib and metformin target cancer cells and their derived stem cells. A potentiating effect of metformin as adjuvant to sorafenib has recently been reported in cholangiocarcinoma cells (26).

Sorafenib and metformin inhibited MAP kinase signaling as demonstrated by reduced ERK phosphorylation. A synergistic effect was, however, not detected. Therefore, the synergistic inhibition of cell growth may be explained by the different targets of the two drugs. Metformin additionally decreases thyroid carcinoma cell growth by inhibition of the AMPK-mTOR pathway and, as mentioned earlier, sorafenib by targeting other receptor-dependent kinases (11).

In conclusion, the multikinase inhibitor sorafenib and metformin synergistically decreased the growth rate of ATC cells. These drugs share a common target in cancer therapy, the MAP kinase pathway. Additionally, each drug inhibits other growth-regulatory signaling pathways and exert their anti-mitogenic effect on the derived cancer stem cells. The synergistic effect of metformin suggests this drug as an adjuvant to sorafenib treatment to reduce dose-dependent side-effects. Clinical studies are necessary to evaluate whether a combined therapy of sorafenib and metformin are useful for the treatment of radioiodine-refractory DTC and anaplastic thyroid cancer in diabetics and other patients with hyperinsulinemia.

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