Abstract. Background/Aim: Beyond their primary field of application some well-established drugs exhibit antitumour effects in a variety of cancers. The aim of this study was to investigate the effects of the COX2 inhibitor celecoxib and the mTOR antagonist rapamycin on angiosarcoma cell lines. Materials and Methods: Cell proliferation was measured in ASM, ISOS 1 and ISO HAS angiosarcoma cell lines with the BrdU assay. Results: In all angiosarcoma cell lines, celecoxib as well as rapamycin inhibited cell growth in a dose-dependent manner. In ASM and ISOS 1, but not in ISO HAS angiosarcoma cells, additive growth inhibitory effects were detected by combining both agents. Conclusion: Our results indicate that angiosarcoma cell proliferation can be inhibited by subtoxic doses of rapamycin and celecoxib. Due to their direct and stroma-mediated anticancer activities, mTOR antagonists and COX2 inhibitors represent very promising drugs in the palliative treatment of angiosarcoma.

Angiosarcoma is a rare and very aggressive malignancy of vascular origin. Cutaneous angiosarcoma shows a clear predilection for the head and neck region (1). Angiosarcoma is usually found in elderly patients. In women with breast cancer, the risk of angiosarcoma was reported to be increased in the irradiated breast after breast-conserving therapy (2, 3). Despite all therapeutic efforts, the prognosis for angiosarcoma patients is poor, with a reported 5-year survival rate of 10% to 30% (4). Only early therapy seems to influence the outcome at all. For this reason, the therapy of angiosarcoma remains a great challenge in dermatology. In recent years, some well-established drugs such as mTOR antagonists and COX2 inhibitors revealed interesting antitumour efficiency (5). Due to their low toxicity, the possibility of an outpatient setting and especially due to their antiangiogenetic potential, the use of these agents in the palliative therapy of angiosarcoma warrants to be investigated.

Celecoxib is a selective inhibitor of cyclooxygenase 2 (COX 2), a key enzyme catalysing the rate-limiting step in the conversion of arachidonic acid to prostaglandins. Three isoforms of COX have been identified. COX1, constitutively expressed in many tissues, is important for the maintenance of homeostatic functions (6). Being an intermediate early response gene, COX2 can be rapidly induced by cytokines, hypoxia and growth factors and is involved in pathological processes like pain sensation and inflammation. Under physiological conditions, COX2 expression is very low or not detectable, but there are also some known exceptions including the brain, kidney and ovarian follicles (7). The existence of COX3 in humans is still under debate (8).

In a variety of cancer types, COX2 overexpression was detected, including colorectal (9), gastric, pancreatic, breast, cervical, non-small cell lung, hepatocellular and prostate cancer (6, 10, 11). Results from several preclinical and clinical trials (12-19) indicate that non steroidal anti-inflammatory drugs (NSAIDs) and especially selective COX2 inhibitors reveal anticancer activities. Beyond direct antitumour effects such as inhibition of cell proliferation and induction of apoptosis, antiangiogenetic and pro-immune surveillance mechanisms as well as modulation of the tumour–stroma interaction are involved in this process. Interestingly, some of the antineoplastic activities of COX2 inhibitors are judged to be independent of the COX2 expression levels (12-15, 20).

The atypical serine/threonine kinase mTOR is a master switch between catabolic and anabolic metabolism and regulates multiple functions such as transcription and translation. Thus, via regulating protein translation by phosphorylation of its two major downstream effectors, the 40S ribosomal protein p70 S6 kinase (S6K1) and the eIF4E binding protein 1 (4E-BP1), mTOR signalling is involved in
the control of cell proliferation, differentiation, migration and survival (21). As a consequence, blocking mTOR function causes growth inhibition, cell cycle arrest and reduction of protein synthesis (21). The activity of mTOR is regulated by the concentration of amino acids, ATP and glucose as well as by growth factors and their receptors. Activating growth factor signalling is transmitted via the IGFR-PI3K-AKT-mTOR pathway, while PTEN and TSC1/2 negatively regulate mTOR function. The first defined inhibitor of mTOR, rapamycin (sirolimus), is a natural fungicide isolated from the solid bacteria Streptomyces hygroscopius by Vezina et al. in 1975 (22). Due to its immunosuppressive activities, rapamycin is used to prevent allograft rejection in organ transplantation. During its preclinical evaluation, rapamycin revealed potent antiproliferative effects in tumour cells (23, 24). This study was designed to evaluate the anticancer activity of celecoxib and rapamycin as single agents or in combination in angiosarcoma cell lines.

Materials and Methods

Reagents and chemicals. Celecoxib (Celebrex®) was a generous gift from Pfizer (Berlin, Germany). A stock solution of celecoxib was prepared by dissolving the drug in 100% dimethyl sulfoxide (DMSO). Commercially available rapamycin (Rapamune®; Wyeth, Münster, Germany) was used as a stock solution. The final concentrations were achieved by diluting rapamycin and celecoxib stock solution in endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 5% FCS, 0.4% ECGS/H, epidermal growth factor 10 ng/ml and hydrocortisone 1 μg/ml. All solutions were prepared freshly prior to use. As celecoxib and rapamycin are bound to plasma proteins, the concentrations required for achieving antiproliferative effects will be lower if the drugs are dissolved in serum-reduced or serum-free media. Since in vivo serum-free conditions cannot be achieved, we decided to perform all experiments with medium containing 5% FCS.

Cell culture. Three angiosarcoma cell lines were used in this study. The human angiosarcoma cell line ASM was kindly contributed by V. Krump-Konvalinkova and C.J. Kirkpatrick (Institute of Pathology, Johannes Gutenberg University Mainz, Germany) (25), the human angiosarcoma cell line ISO HAS and the murine-phenotypic angiosarcoma cell line ISOS 1 were kindly contributed by M. Masuzawa (Department of Dermatology, Kitasato University School of Medicine, Sagamihara, Japan) (26, 27). For all angiosarcoma cell lines an endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 5% FCS, 0.4% ECGS/H, epidermal growth factor 10 ng/ml and hydrocortisone 1 μg/ml was used. All solutions were prepared freshly prior to use. As celecoxib and rapamycin are bound to plasma proteins, the concentrations required for achieving antiproliferative effects in vitro are lower if the drugs are dissolved in serum-reduced or serum-free media. Since in vivo serum-free conditions cannot be achieved, we decided to perform all the experiments with medium containing 5% FCS.

Cell proliferation. The effects of rapamycin, celecoxib and the combination of both on angiosarcoma cell growth were detected by using the cell proliferation ELISA BrdU (Roche applied science, Mannheim, Germany) as previously described (28). In brief, cell proliferation was quantified by measuring BrdU incorporation during DNA synthesis. Cells (2-3x10^5) were plated in 96-well plates (Costar, Bodenheim, Germany) and incubated with the reagents for 48 h and for 16 h with BrdU labelling solution. After fixing the cells, denaturating DNA, incubating with Anti-BrdU-POD solution for 90 min, washing and incubating with TMB substrate for 15 min, immune complexes were detected by measuring absorbance at 405 and 490 nm. Eight wells per treatment group were used and all tests were repeated at least twice. Drug concentration ranges were chosen according to recent results with melanoma cell lines (28) and to the specific literature available (12, 29-31).

Statistical analysis. Results are expressed as mean values±SD. For comparison between mean values the non-parametric Mann-Whitney U-test was applied. As cell proliferation was calculated relative to the untreated control, one-sided statistical tests were used. Differences were considered statistically significant at p<0.05.

Results

Rapamycin and celecoxib inhibit angiosarcoma cell proliferation. The angiosarcoma cell lines ASM, ISO HAS and ISOS 1 were incubated with increasing concentrations of rapamycin (0.025-1 μM) and celecoxib (1-60 μM) for 48h. The effects on cell proliferation were detected by measuring BrdU incorporation during DNA synthesis. In all angiosarcoma cell lines, celecoxib as well as rapamycin inhibited cell growth in a dose dependent manner. In the human angiosarcoma cell lines ASM and ISO HAS cell proliferation was reduced by concentrations of rapamycin ranging from 0.025 to 0.2 μM (Figure 1A and B). However, to achieve comparable growth inhibitory effects in the lower differentiated murine angiosarcoma cell line ISOS 1, higher concentrations of rapamycin (0.05-1 μM) were necessary (Figure 1C). In ASM and ISO HAS angiosarcoma cell lines, 0.05 μM rapamycin reduced cell proliferation by 39% and 46% respectively while in ISOS 1 angiosarcoma cell line only a reduction of 11% could be detected using the same concentration of rapamycin.

Treatment with 1-60 μM celecoxib caused a significant inhibition of cell growth in all angiosarcoma cell lines. For example, 50 μM celecoxib inhibited cell proliferation in ISO HAS, ASM and ISOS 1 angiosarcoma cell lines by 78%, 61% and 69% respectively (Figure 2).

Combinatorial treatment causes additive antiproliferative effects. In a recent study, we observed additive growth inhibitory effects in melanoma cell lines by combining both agents (28). We were interested to investigate, whether a combinatorial treatment with rapamycin and celecoxib would also exert stronger growth inhibitory effects in angiosarcoma cell lines compared to the single-drug treatment. To detect potential additive effects, drug concentrations which only caused a slight reduction of cell proliferation when administered as single agents were chosen. In ASM and ISO HAS cells, 0.025 μM rapamycin was combined with 20 μM
celecoxib, and in the ISOS 1 angiosarcoma cell line, 0.2 μM rapamycin and 20 μM celecoxib were used. By combining both agents, in ASM and ISOS 1 but not in ISO HAS angiosarcoma cell lines, additive growth inhibitory effects were detected (Figure 3). While rapamycin and celecoxib reduced angiosarcoma cell proliferation by 23% and 20% in ASM cells and by 19% and 38% in ISOS 1 cells, the combinatorial treatment inhibited cell proliferation at a significantly higher rate (38% in ASM and 54% in ISOS 1) (p<0.05).

Discussion

The results of this study indicate that celecoxib and rapamycin inhibit angiosarcoma cell growth in a dose-dependent fashion. In ASM and ISOS 1 angiosarcoma cell lines, the combinatorial treatment with both agents caused additive antiproliferative effects. In recent years, targeting mTOR for cancer therapy has...
attracted more and more attention. For this reason, we investigated the effects of rapamycin on angiosarcoma cell proliferation. Rapamycin caused a reduction of cell proliferation in all angiosarcoma cell lines in a dose-dependent manner. However, in the less-differentiated murine angiosarcoma cell line ISOS 1, higher concentrations of rapamycin were necessary to achieve growth inhibitory effects comparable to that of the human angiosarcoma cell lines ASM and ISO HAS. Recently, we observed a dose-dependent growth inhibition in melanoma cell lines by concentration of rapamycin ranging from 0.2-1 μM (28). Similar results were found in mammary tumours (29), while in other tumour entities, even nanomolar concentrations of rapamycin caused comparable reduction of cell proliferation (32, 33). Some investigators postulated that blocking mTOR function may be most potent in tumours containing PTEN mutation. As PTEN is an important negative regulator of mTOR, mTOR function is enhanced in tissue containing PTEN abnormalities and tumour cells are hypersensitized to mTOR antagonists (34). To date, two studies investigated the PTEN status in angiosarcoma. A patient with PTEN germline mutation developed hepatic angiosarcoma (35) and in canine hemangiosarcoma somatic point mutations or deletions encompassing the PTEN C-terminal domain could be detected (36). The PTEN status of the angiosarcoma cell lines used in this study is unknown.

The knowledge of the antitumour effects of mTOR antagonists is not limited to the results of preclinical studies. Currently, several clinical trials using rapamycin or analogues are ongoing or have already been finished, showing encouraging antineoplastic effects and low toxicity profiles (37-39). Compared with the general population, the incidence of Kaposi’s sarcoma is increased in kidney transplanted patients. In one study, 15 renal graft recipients who had developed a Kaposi sarcoma were treated with sirolimus. After a 3-month treatment, all cutaneous Kaposi sarcomas had disappeared (40). The findings of several other studies have confirmed the regression of Kaposi’s sarcoma after conversion to sirolimus in kidney transplant recipients (41, 42). In addition, therapy with temsirolimus (CCI-779) in patients with recurrent glioblastoma multiforme was associated with significantly longer time to progression and radiographic improvement was observed in 36% of the patients (43). In a double-blind, randomised placebo-controlled phase III trial treatment with everolimus, prolonged progression-free survival was observed in patients with advanced renal cell carcinoma (44). However, monotherapy with mTOR antagonists lacked efficiency in some tumour entities such as metastatic melanoma (45). As treatment with these agents cause low toxicity, mTOR antagonists may merit further investigation in combination with other biomodulators or cytotoxic agents. In ovarian, endometrial and scirrhus gastric cancer combinatorial treatment with mTOR antagonists and chemotherapeutic drugs potentiated antineoplastic effects by increasing growth inhibition and apoptosis (46-48).

COX2 is involved in carcinogenesis and supports tumour growth and metastasis via different mechanisms such as promoting the conversion of pro-carcinogens to carcinogens, the generation of free radicals, facilitating the escape from host surveillance mechanisms and inducing resistance to apoptotic cell death (49). Blocking COX2 function by selective COX2 inhibitors was shown to inhibit cell proliferation and to induce cell cycle arrest and apoptosis in preclinical trials (12-15). The clinical trials using celecoxib in combination with other biomodulating or cytotoxic agents showed encouraging results (16, 18, 19, 50, 51). Sheu et al. detected a strong COX2 expression in 20% of the investigated thyroid angiosarcoma samples. Although celecoxib failed to inhibit tumour progression in thyroid carcinoma, in two cases a partial or complete remission related to tumours with immunohistochemically proven strong COX2 expression was observed (52). However, as some of the anticancer activities achieved by coxibs are judged to follow COX2 independent pathways, the efficiency of coxibs in cancer therapy do not necessarily depend on a high COX2 expression level in the targeted tissue. Waskewich et al. observed no difference between anti proliferative effects of celecoxib in COX2 positive and negative endothelial cell lines (53). These findings agree with the results from several preclinical studies investigating either cell lines with low baseline COX2 expression (13, 20, 28), COX2-deficient cell lines (14, 15) or silencing COX2 activity by antisense depletion (12).

In several studies, rapamycin or celecoxib were combined with other biomodulating or cytotoxic agents (48, 51, 54-56). In combination with sorafenib or an inhibitor of the MSPK kinase 1, rapamycin was effective in inhibiting tumour cell growth in vivo and in vitro, and reduced lung metastasis and enhanced apoptosis rate in prostate and hepatocellular carcinoma (57, 58). Furthermore, celecoxib potentiated the antitumour effect of chemotherapeutic drugs currently used in neuroblastoma treatment (59).

To our knowledge, the effects of a combination of rapamycin and celecoxib on angiosarcoma cell lines have not previously been examined. According to our recent findings in melanoma cell lines, we hypothesized that a combinatorial treatment with both agents would cause stronger antitumour activity than monotherapy. Indeed, we detected a significant enhancement of the antiproliferative effects in ASM and ISOS 1 cells but not in ISO HAS angiosarcoma cell line. These results encourage further investigation of the anticancer potential of biomodulating drugs in angiosarcoma. The idea of treating angiosarcoma with COX2 inhibitors has already entered clinical trials. A triple therapy with the COX2 inhibitor refocoxib, the PPARγ agonist pioglitazone and metronomic scheduled trofosfamide revealed efficiency...
in the treatment of advanced and pre-treated malignant angiosarcoma (18). Four out of the six patients enrolled in this study showed a clinical benefit (2 complete remissions, 1 partial remission, and 1 stable disease >6 month). The median progression-free survival was 7.7 months. The same triple therapy regimen prolonged stabilization of disease in patients with chemorefractory malignant melanomas and soft tissue sarcomas (16). Interestingly, this therapy also revealed efficiency in a case of endemic Kaposi sarcoma (17) and chemorefractory multisystem Langerhans’ cell histiocytosis (60), indicating a certain ‘universality’ of this approach by targeting pathways common in many tumours.

In conclusion, we show that COX2 inhibitors and mTOR antagonists are potent inhibitors of angiosarcoma cell growth in vitro. Taking into account the results of the clinical trials, combinatorial treatment with biomodulating drugs seem to be a promising strategy in tumour palliation. Further experimental and clinical studies are necessary to optimise the anticaner potential of these agents in angiosarcoma.

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References


51 Gadgeel SM, Wozniak A, Ruckdeschel JC, Heilbrun LK, Venkatramanamoorthy R, Chaplen RA, Kraut MJ and


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