Abstract. Inhibitors of cyclooxygenase 2 (COX 2) and the mammalian target of rapamycin (mTOR) show direct and indirect antitumor effects in a variety of cancers. This study was designed to investigate the effects of the mTOR antagonist rapamycin and the COX 2 inhibitor celecoxib on cell growth and apoptosis in malignant melanoma. Cell proliferation was analysed by the cell proliferation ELISA BrdU and alamarBlue assay and apoptosis was measured by caspase 3 and 7 activity in two out of six melanoma cell lines (A375 and Mel Ho) that were selected for the heterogeneous levels of the COX 2 mRNA expression. The quantitative real-time reverse transcription polymerase chain reaction showed a 337-fold higher COX 2 mRNA level in the A375 than in the Mel Ho melanoma cells. However, both celecoxib and rapamycin caused significant growth inhibition in the two cell lines. By combining both agents, additive growth inhibitory effects were observed in the A375 cells. Treatment with celecoxib, but not rapamycin, increased apoptosis in the two cell lines. Our data indicate that rapamycin and celecoxib inhibit melanoma cell growth as single agents and a combination of both drugs have additive antitumor effects. Notably, the antiproliferative and proapoptotic effects of celecoxib seem to be independent of the COX 2 expression. Both rapamycin and celecoxib represent promising drugs for the palliative therapy of metastasised malignant melanoma and should be considered for future trials.

Introduction

In recent years, the incidence and mortality of malignant melanoma has continued to rise. While the early stages of the disease can often be cured by appropriate therapy, advanced and metastasised malignant melanoma is characterised by a short median survival time and implies a poor prognosis. For this reason the treatment of advanced melanoma remains a challenge in dermatoncology. In the search for new strategies in tumor palliation, some well-established drugs such as rapamycin and celecoxib were shown to reveal broad antitumor activities (1) and, given their low toxicity their potential for palliative therapy of malignant melanoma seems to be grossly under-researched.

Rapamycin (sirolimus) is a natural fungicide isolated from the soil bacteria *Streptomyces hygroscopius*. Due to its profound immunosuppressive actions, rapamycin is used to prevent allograft rejection in organ transplantation. However, during its preclinical evaluation, rapamycin also revealed antitumor activities (2). As a complex combined with immunophilin FKBP 12, rapamycin inhibits the action of the mammalian target of rapamycin (mTOR), an atypical serine/threonine kinase playing a key role in the regulation of cell proliferation, differentiation, migration and survival (3). This kinase phosphorylates regulators of translation such as p70-S6 kinase and the eukaryotic translation initiation factor, 4E binding protein 1 (4E-BP1). Consequently, the blocking of mTOR results in cell cycle arrest, growth inhibition and a reduction of protein synthesis (3). The immunosuppressive potency of rapamycin is based on the inhibition of the lymphocyte proliferation stimulated by IL-2. The activity of mTOR is regulated by a concentration of amino acids, ATP and glucose. Activating growth factor signaling is transmitted via the IGFR-PI3K-AKT-mTOR pathway while TSC1/2 and PTEN are negative regulators of the mTOR function.

COX 1 and 2 are isoforms of the cyclooxygenase, a key enzyme in the conversion from arachidonic acid to prostaglandins. COX 1 is constitutively expressed in many tissues and catalyses the physiiological prostaglandin synthesis. COX 2 is an immediate early response gene involved in pathological processes like inflammation and pain sensation. Under normal conditions, the COX 2 expression is low or not detectable in most tissues, but some exceptions such as the kidney and CNS are also known (4). COX 2 overexpression was found in a variety of human malignancies, including colorectal, head and neck, pancreatic, cervical, breast, gastric cancer (5) and malignant melanoma (6). In several clinical (7-11) and preclinical trials (12-15), NSAIDs and especially selective COX 2 inhibitors revealed anticancer activities.
These effects are based on direct antitumor effects such as the induction of apoptosis and inhibition of tumor growth as well as on the modulation of the tumor-stroma interaction and inhibition of angiogenesis. Notably, some of the antitumor activities of COX 2 inhibitors seem to follow COX 2-independent pathways (12,15,16).

The aim of this study was to elucidate the antitumoral potential of rapamycin and the selective COX 2 inhibitor celecoxib administered singly or combined in malignant melanoma cells.

Materials and methods

Reagents and chemicals. Commercially available rapamycin (Rapamune®, Wyeth, Münster, Germany) was used as a stock solution. Celecoxib (Celebrex®) was a generous gift from Pfizer. A stock solution of celecoxib was prepared by dissolving the drug in 100% dimethyl sulphoxide (DMSO). The final concentrations were achieved by diluting rapamycin and celecoxib stock solution in RPMI medium containing 10% fetal calf serum (FCS), penicillin-streptomycin (PS) and L-glutamine (LG) (Gibco). 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 no serum-free conditions can be achieved, we decided to perform all the experiments with a medium containing 10% FCS.

Cell culture. Six human melanoma cell lines were screened for this study. A375 cells were purchased from the American Type Culture Collection and Mel Ho, Colo 849 and IGR 1 cells were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). The nude-mouse selected isogenic variants of the A375 cell line with strong (SM) and poor (P) metastatic potential were kindly contributed by K. Gehlsen (S.K.C.C., San Diego, CA, USA) (17). A375, A375 P, A375 SM, Mel Ho and Colo 849 were grown in an RPMI-1640 formulation supplemented with 10% FCS (20% for Colo 849), PS and LG (Gibco) at 37°C in a humidified CO2 atmosphere (5 and 8%). For IGR 1 cells, Dulbecco’s modified Eagle’s medium (DME) supplemented with 10% FCS, PS and LG was used. Cells were cultured in Falcon 75-cm2 tissue culture flasks and were maintained in a monlayer culture. Experiments were performed when cells reached ~80% confluence.

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Six melanoma cell lines were screened for COX-2 mRNA expression. Cells were lysed by adding RLT buffer (Qiagen), released by scratching and lysates were homogenized by using Qia-shredder columns (Qiagen). RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA concentration was measured spectrophotometrically at 260 nm and RNA was stored at -80°C. Total RNA was used as a template for cDNA synthesis according to the instructions of the Reverse Transcription System Kit (Promega). For quantification of the COX-2 transcripts TaqMan RT-PCR was performed as previously described (18,19).

Cell proliferation. Proliferation of cells treated with celecoxib, rapamycin or the combination was determined using two different methods.

The alamarBlue™ assay (Biosource) is a colorimetric growth indicator based on the detection of metabolic activity. Cells were seeded into 24-well plates (Falcon) at a cell density of 1.2x10⁴ (A375) or 1.5x10⁴ (Mel Ho) per well in the standard growth medium and were incubated for 24 h at 37°C to allow re-attachment. Then the culture medium was removed and replaced with 900 μl of the test compounds (rapamycin, celecoxib or combination) or vehicle control (DMSO or RPMI) in various concentrations. AlamarBlue assay (100 μl) was added into each well and the plates were cultured for 48 h. After 24 and 48 h of treatment absorbance at 540 and 600 nm was measured using the Mithras multilabel reader (Berthold Technologies). All tests were performed in quadruplicate and repeated at least twice.

In addition, the BrdU (Roche) immunoassay was used to measure BrdU incorporation during DNA synthesis. Cells (2-3x10⁴) were plated in 96-well plates (Costar) and incubated overnight at 37°C. Then the cells were treated with 100 μl of the test compounds and put back into the incubator for 32 h before being treated with 10 μl of BrdU labelling solution for another 16 h. After an overall treatment time with the test compounds of 48 h, the medium was removed. The cells were incubated for 30 min with 200 μl FixDenat solution at room temperature for fixing the cells and denaturating DNA and then for 90 min with antiBrdU-POD solution. Antibody conjugates were removed by washing three times with 200 μl of washing buffer. After incubation with a TMB substrate for 15 min, immune complexes were detected by measuring the 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 the specific literature available (13,20,21).

Apoptosis analysis. Cells (10⁴) were seeded into a 75-cm² tissue culture flask (Falcon) in the standard growth medium and were allowed to adhere for 24 h before being treated with celecoxib (50 μM) and/or rapamycin (0.5 μM) or vehicle (DMSO or RPMI) for 24 h. For apoptosis analysis, the activity of caspase 3 and 7 was measured by a Caspase-Glo™ 3 and 7 assay (Promega). The cells were released by scratching and re-suspended in serum-free medium, counted and seeded into a white 96-well multiliter plate (10⁴ cells in 100 μl serum-free media/well). Eight wells per treatment group were used. After equilibration to room temperature, 100 μl of Caspase-Glo 3 and 7 assay were added to each well. The plate was incubated at room temperature and protected from light for 1 h. Finally, luminescence was measured using the Mithras multilabel reader and Mikro-Win Software.

Cell cycle analysis. Cell cycle profiles were measured by flow cytometry. A375 and Mel Ho cells were cultured with celecoxib (50 μM) and/or rapamycin (0.5 μM) or vehicle for 24 h. The cells were released by treatment with trypsin-EDTA, counted (10⁴ cells), washed twice with cold PBS/5 mM EDTA and fixed at room temperature with 100% ethanol for 30 min. After washing again with PBS/5 mM EDTA, the cells were treated with 1 mg/ml RNase A and incubated for another
30 min at room temperature. Finally, the cells were stained with 100 μg/ml propidium iodide (Sigma) and analysed by flow cytometry using FACSCalibur and Cellquest software. For each sample 10^4 fluorescent cells were counted.

Statistical analysis. The results are expressed as means ± SD. For comparisons between the means, the non-parametric Mann-Whitney U test was applied. Cell proliferation was calculated relative to the untreated control which was designated at 100%, whereas the data obtained by apoptosis and cell cycle analysis were compared directly. For this reason, the statistical tests were 1-sided for the cell proliferation experiments and 2-sided for the apoptosis and cell cycle analysis. Differences were considered statistically significant at p<0.05.

Results

COX 2 mRNA expression in A375 and Mel Ho melanoma cell lines. To analyse a possible dependence of the COX 2 expression on the effectiveness of coxibs we first screened six melanoma cell lines (A375, A375 SM, A375 P, Mel Ho, IGR 1 and Colo 849) for their COX 2 mRNA expression level using the quantitative RT-PCR (Fig. 1). The COX 2 expression was detectable in all melanoma cell lines. The COX 2 mRNA level was highest in A375, followed by A375 SM and Colo 849 cell lines. In the IGR 1, A375 P and Mel Ho melanoma cell lines COX 2 expression was very low. To determine whether the effects of the COX 2 inhibitors were influenced by the COX 2 expression, we selected A375 (high expression) and Mel Ho (low expression) melanoma cell lines for further investigation. In the A375 cells the COX 2 mRNA expression was 337-fold higher than in the Mel Ho melanoma cells. A similar analysis for mTOR was not possible since no quantitative tool is currently available.

Celecoxib and rapamycin inhibit proliferation in melanoma cell lines. The A375 and Mel Ho cell lines were incubated with increasing concentrations of rapamycin (0.2-1 μM), celecoxib (10-100 μM) or a combination of both drugs (50 μM celecoxib and 0.5 μM rapamycin) for 48 h. The effects on cell proliferation were measured by the alamarBlue assay, a colorimetric growth indicator based on the detection of metabolic activity. Celecoxib caused a significant, dose-dependent inhibition of proliferation in both cell lines (Fig. 2A and B). For example, 80 μM celecoxib significantly reduced cell proliferation in A375 cells by 78% compared to the untreated control. Similarly, rapamycin (0.5 μM) caused a 35% reduction in cell proliferation in Mel Ho cells.

Figure 2. Dose-dependent growth inhibition by celecoxib and rapamycin. Mel Ho and A375 melanoma cells were grown overnight in a 24-well plate and then treated with increasing concentrations of celecoxib and rapamycin for 48 h. Cell proliferation was measured using the alamarBlue assay and was calculated relative to the untreated control (DMSO for celecoxib and standard growth medium for rapamycin-treated cells). (A) A375 treated with celecoxib, (B) Mel Ho treated with celecoxib, (C) A375 treated with rapamycin and (D) Mel Ho treated with rapamycin; *p<0.001 compared with the respective control culture.
cell growth (by 31±6 in Mel Ho and 37±6% in A375). Thus the antiproliferative effects were comparable in the two melanoma cell lines regardless of the difference in the COX 2 expression level. Treatment with rapamycin also resulted in a dose-dependent growth inhibition. Rapamycin (1 μM) reduced A375 and Mel Ho cell proliferation by 36±4 and 39±8%, respectively (Fig. 2C and D). At lower concentrations, targeting the mTOR was more effective in the Mel Ho than in the A375 melanoma cell line. To confirm the data obtained by the alamarBlue assay, we used the cell proliferation ELISA BrdU assay. We detected very similar growth inhibitory effects (data not shown). In several preclinical studies mTOR antagonists and COX 2 inhibitors showed encouraging results when used in combination with other anticancer agents. For this reason we were interested in whether a combinatorial treatment of rapamycin and celecoxib would exert stronger antiproliferative effects on melanoma cell proliferation than each drug alone. To determine potential additive or even super-additive effects, we chose drug concentrations which only caused slight growth inhibition when administered as single agents. By combining 50 μM celecoxib with 0.5 μM rapamycin, a significant increase in growth inhibition was detected in the A375 cells (Fig. 3A). While rapamycin and celecoxib reduced melanoma cell proliferation by 19±8 and 13±6% respectively, the combinatorial treatment inhibited proliferation at a significantly higher rate (36±4%) (p=0.002). In the Mel Ho cells no additive growth inhibitory effects were detected (Fig. 3B).

Celecoxib induces apoptosis in melanoma cell lines. In order to ascertain if the inhibition of cell proliferation is associated with an enhanced apoptosis rate, we measured the activity of caspase 3 and 7 after incubation using the same concentration as mentioned above, i.e., 0.5 μM rapamycin and/or 50 μM celecoxib for 24 h (Fig. 4). Celecoxib significantly increased caspase activity in the A375 and Mel Ho cell lines by 1.4- and 2.2-fold, respectively, when compared with the untreated vehicle control. Rapamycin caused a slight enhancement of apoptosis, which only reached a significant level in the Mel Ho cells (1.1- and 1.3-fold, respectively). By combining rapamycin and celecoxib, a modest but significant protection against apoptosis were observed in the two cell lines.

Rapamycin but not celecoxib induces cell cycle arrest. To elucidate the mechanism underlying the growth inhibition, we performed a cell cycle analysis. In the A375 cells rapamycin caused an increase in cells in the G-1 phase from 56.5±4.1 to 67±2.0% compared to the untreated control, accompanied by a decrease in the G-2 phase from 31.4±1.3 to 23.4±1.9% (Fig. 5). In Mel Ho the increase from 56.5±3.5 to 59.5±2.1%
Table I. Cell cycle analysis.

<table>
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<tr>
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<th>Mel Ho</th>
<th>A375</th>
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<tr>
<td></td>
<td>G-1</td>
<td>S</td>
</tr>
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<td>Medium</td>
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<td>15.7±1.2</td>
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<td>10.6±0.0</td>
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<tr>
<td>DMSO</td>
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<tr>
<td>Celecoxib (50 μM)</td>
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<td>Combination</td>
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<tr>
<td>G-1</td>
<td>56.5±4.1</td>
<td>11.9±3.2</td>
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<tr>
<td>S</td>
<td>67.0±2.0</td>
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<tr>
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<tr>
<td>Rapamycin (0.5 μM)</td>
<td>62.1±2.5</td>
<td>11.8±1.1</td>
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Cell cycle distribution of the Mel Ho and A375 cells after treatment for 24 h with rapamycin (0.5 μM), celecoxib (50 μM), a combination or control (medium for rapamycin and DMSO for celecoxib-treated cells). Data are presented as means (%) ± SD.

Discussion

In this study we demonstrated that celecoxib and rapamycin inhibit melanoma cell proliferation in a dose-dependent fashion and that celecoxib but not rapamycin induce significant apoptosis. The growth inhibitory effects in celecoxib seem to be independent of the COX 2 baseline expression in the melanoma cells. A combinatorial treatment with both agents resulted in the additive inhibition of cell growth in the A375 melanoma cells, although this effect was accompanied by a decrease of caspase activity.

COX 2 is involved in carcinogenesis and promotes tumor cell invasion and metastasis. COX 2 overexpression was found in a variety of human malignancies, including colorectal, head and neck, pancreatic, cervical, breast, gastric cancer (5) and malignant melanoma (6). In this study, COX 2 mRNA was differentially expressed in six melanoma cell lines. The significance of the COX 2 overexpression in melanoma pathogenesis is still controversial, but the expression seems to vary considerably in malignant melanoma and metastasised malignant melanoma (6,16,22). This raised the question of whether low COX 2-expressing cell lines would be less susceptible to coxibs. To answer this question, we chose A375 as high and Mel Ho as low COX 2-expressing cell lines for further investigation. In both cell lines, celecoxib inhibited proliferation in a dose-dependent fashion and induced apoptosis by enhancing the caspase 3 and 7 activity. The growth inhibitory effects were comparable in the two cell lines and the enhancement of the caspase 3 and 7 activity was stronger in the Mel Ho melanoma cells. These data indicate that the anticancer effects of celecoxib do not depend on the COX 2 expression in melanoma. These findings agree with the results of a previous study comparing the effects of sulindac sulphide on the apoptosis of COX 2-negative melanoma and COX 2-positive colon carcinoma cells. As there was no significant difference in the rate of apoptotic cell death, NSAID-induced apoptosis was judged to follow COX 2 independent pathways (16). Similar results were obtained in several tumor entities by investigating either COX 2-deficient cell lines (12,15), cell lines with a low baseline COX 2 expression (21) or silencing COX 2 activity by antisense depletion (13). In addition, it was reported that NSAIDs reveal their antitumor potency only at doses that are >100- to 1000-fold than those required to block the prostaglandin synthesis by inhibition of the COX 2 activity (23).

In this study we detected the inhibition of cell proliferation with a broad range of concentrations of celecoxib (10 to 100 μM). Regarding apoptosis, a concentration of 50 μM of celecoxib induced an enhancement of caspase 3 and 7 activity in the two cell lines while no changes in cell cycle distribution could be measured. The induction of apoptosis and cell cycle arrest are effects often observed in cells treated with celecoxib, whereas the concentrations required to achieve these effects vary among different cancer types (12,13,15). Reportedly, some mechanisms underlying coxib-induced apoptosis have already been defined. Dandekar et al observed an augmentation of chemotherapeutic-drug-induced apoptosis in prostate cancer cells via the activation of caspase 3 and 9, decreased levels of Bcl-xl and increased BAD concentration (24). In non-small cell lung cancer celecoxib-triggered apoptosis was shown to be based on the activation of death receptor 5 (25). Furthermore, a reduction of protein kinase B/AKT signaling was described after treatment with celecoxib (26). In melanoma the induction of FasL-mediated
death signaling by coxibs was observed (27). In the clinical trials celecoxib in combination with other anticancer agents showed encouraging results without causing significant toxicities (7-11).

Rapamycin has emerged as a more recent biomodulator in cancer. Therefore, we further investigated the effects of mTOR antagonists on the proliferation, apoptosis and cell cycle of those melanoma cell lines. The mTOR-antagonist rapamycin was effective in inhibiting melanoma cell proliferation in a dose-dependent fashion. Treatment with 1 μM rapamycin reduced A375 and Mel Ho cell growth by 36 and 39%, respectively. While similar results were found in mammary tumors, both, in other cancers even nanomolar concentrations of rapamycin lead to comparable antiproliferative effects (28,29). Some investigators have postulated that targeting the mTOR may be most potent in tumors containing PTEN mutations.

In melanoma, a loss of the PTEN function is a late step in melanoma tumorigenesis. Accordingly, a PTEN-mutation rate of 30-50% was detected in melanoma cell lines whereas only 10% of primary melanomas exhibit PTEN alterations (30). In the A375 cell line no alterations in the PTEN status were found (31). This may explain the high doses of rapamycin required to achieve growth inhibition in this particular melanoma cell line in comparison with other tumor types. The PTEN status of Mel Ho has yet to be determined.

In addition to the antiproliferative effects, 0.5 μM rapamycin induced a certain degree of arrest in the G-1 phase of the cell cycle in the A375 cells plus a modest enhancement of the caspase 3 and 7 activity. Our findings partly confirm other preclinical studies showing an increasing number of cells in the G-1 phase of the cell cycle by the up-regulation of p21, p27 and CDK1 together with the down-regulation of cyclin A and cyclin D1/CDK 4 levels (28). Accordingly, rapamycin blocked cell cycle progression without inducing apoptotic cell death in renal and non-small cell lung cancer (32,33). Some investigators described a correlation between an enhanced bcl-2 expression or activation of AKT in cancer cells and protection against rapamycin-induced apoptosis (34,35). In contrast, in anaplastic large cell lymphoma cells and protection against rapamycin-induced apoptosis whereas apoptosis by doxorubicin was not affected (34). Romano et al even described an enhancement of the cytolytic effects of doxorubicin in melanoma cells (38). Combinations of celecoxib and chemotherapeutic agents or the tyrosine kinase inhibitor emodin resulted in an augmentation of apoptotic cell death (24,40).

In conclusion, a potential benefit of the combinatorial use of coxibs and mTOR inhibitors emerges that may be even more pronounced if stroma-targeting antitumor effects such as antiangiogenesis are taken into account. These effects need to be determined in future experimental studies.

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References