The Cyclooxygenase-2 Inhibitor Nimesulide, a Nonsteroidal Analgesic, Decreases the Effect of Radiation Therapy in Head-and-Neck Cancer Cells

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Background: No data are available on the effects of the cyclooxygenase-2 (COX-2) inhibitor nimesulide in combination with irradiation on the survival of head-and-neck carcinoma cells.
Material and Methods: Two head-and-neck carcinoma cell lines (SCC9 and SCC25) were treated with nimesulide (50–600 µM) and irradiated concomitantly or sequentially. Early effects on cell survival were investigated by counting cell numbers, long-term effects by colony-forming assays. Cell-cycle effects were analyzed 24–72 h after treatment with nimesulide by flow cytometry.
Results: Unexpectedly, nimesulide solely inhibited cell proliferation without affecting colony-forming ability. In addition, no evidence for a radiosensitizing effect of nimesulide in short-term assays was seen. Nimesulide alone had no effect on clonogenic survival alone or in combination with radiation.
Conclusion: Nimesulide differentially affects cell proliferation and clonogenic survival and may decrease the efficacy of radiotherapy. Short-term assays to assess tumor growth may not correctly predict the clinically relevant long-term effect of COX-2 inhibitors.

Key Words: Cyclooxygenase-2 (COX-2) · Head-and-neck cancer · Nimesulide · Radiation

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Der Cyclooxygenase-2-Inhibitor Nimesulid, ein nichtsteroidales Analgetikum, vermindert den Effekt von Strahlentherapie in Kopf- und Halstumorzellen

Hintergrund: Bis dato gibt es keine Untersuchungen bezüglich der Anwendung des Cyclooxygenase-2-Hemmers Nimesulid in Kombination mit Bestrahlung auf Zellen des Kopf-Hals-Bereichs.
Material und Methodik: Zwei Zelllinien des Kopf-Hals-Bereichs (SCC9 und SCC25) wurden mit Nimesulid (50–600 µM) behandelt und zeitversetzt oder konkomitant bestrahlt. Die Kurzzeiteffekte auf das Überleben der Zellen wurden mittels Zellzählung untersucht, Langzeiteffekte via Koloniebildungsassays. Mittels Durchflusszytometrie wurden die Auswirkungen auf den Zellzyklus 24, 48 und 72 h nach Behandlung evaluiert.
Ergebnisse: Nimesulid allein war in der Lage, die Zellproliferation kurzfristig zu hemmen (Abbildungen 1, 2 und 4), allerdings ohne nachhaltigen Effekt auf die Koloniebildungsfähigkeit (Abbildungen 3 und 5). Darüber hinaus konnte weder in den Kurzzeit- (Abbildungen 2 und 4) noch in den Langzeituntersuchungen (Abbildungen 3 und 5) ein die Strahlentherapie unterstützender Effekt nachgewiesen werden.
Schlussfolgerung: Nimesulid hat unterschiedliche Effekte auf die Zellproliferation und die Koloniebildungsfähigkeit und könnte die Effizienz der Strahlentherapie schwächen. Weiters bestätigt sich, dass aus Kurzzeitanalysen des Tumorwachstums nicht automatisch auf das klinisch relevante Langzeitergebnis geschlossen werden darf.

Schlüsselwörter: Cyclooxygenase-2 (COX-2) · Tumoren des Kopf-Hals-Bereichs · Nimesulid · Bestrahlung

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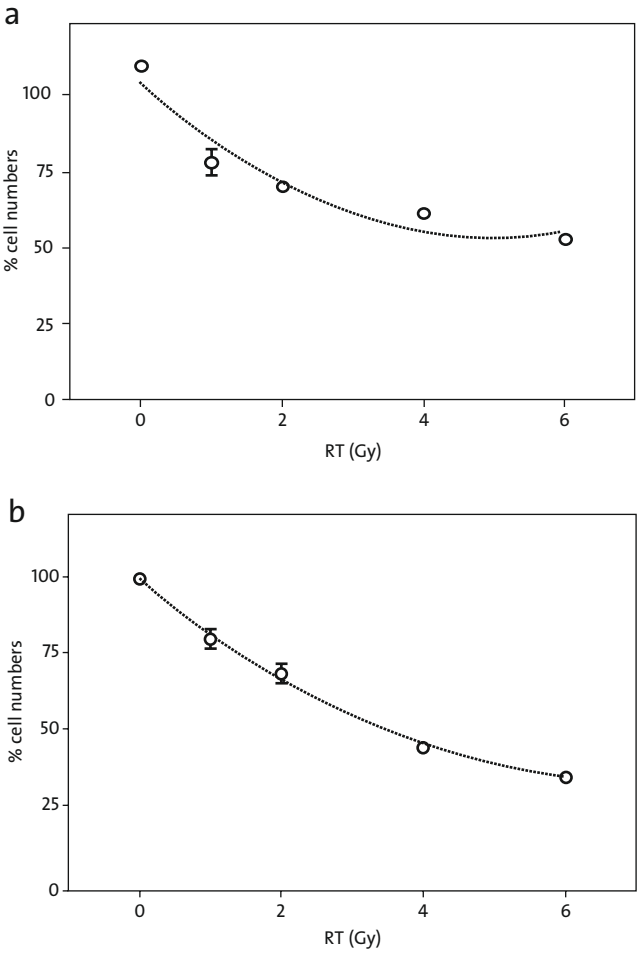
Introduction

Cyclooxygenase (COX) inhibitors are widely used as supportive nonsteroidal analgesics in cancer patients. This class of drugs has mild analgesic, antipyretic and antiphlogistic properties. COX is a key enzyme that catalyzes the conversion of arachidonic acid into prostaglandins. There are two important isoforms of this enzyme, designated COX-1 and COX-2 [30, 32]. COX-1 is thought to be constitutively expressed in most tissues and mediates the synthesis of basal levels of prostaglandins, thus regulating and maintaining various physiological functions such as production of protective mucus by the gastrointestinal mucosa and platelet aggregation. In contrast to COX-1, COX-2 is expressed at low levels under normal physiological conditions. It is known that COX-2 is inducible by inflammatory stimuli, growth factors or oncogenes [23].

COX-2 has been suggested to contribute to carcinogenesis by several mechanisms, for example through xenobiotic metabolism, inhibition of apoptosis and treatment-induced cell death, immunosuppression, stimulation of angiogenesis, invasion and metastasis [5, 7]. Constitutive expression of COX-2 has been described in different types of tumors, especially in squamous cell carcinoma of the head and neck (HNSCC) [4, 11, 14]. It could be shown that selective COX-2 inhibitors have the potential to prevent carcinogenesis and even reduce the growth rate of tumor cells [37, 44].

The exact mechanisms responsible for the antitumor effects of COX-2 inhibitors have not been clearly defined and several of them have been proposed [9, 15, 20, 27, 46]. Inhibition of programmed cell death could be one explanation, since overexpression of COX-2 has been shown to inhibit apoptosis in a variety of cellular systems [24, 43, 45]. In agreement with this hypothesis, treatment with COX-2 inhibitors was found to induce apoptosis in tumor cells [17, 18, 26]. COX-2-independent apoptosis-inducing effects of selective COX-2 inhibitors have also been implicated in the induction of cell death [11, 13, 29]. Experimental evidence suggests that selective COX-2 inhibition might lead to enhanced radiation sensitivity [48]. Preclinical experimental studies suggest that the efficacy of radiation is improved when used in combination with COX-2 inhibitors, though not all types of COX-2 inhibitors have been investigated. In recent reviews, the rational and experimental foundations supporting the use of COX-2 inhibitors with radiotherapy (RT) have been discussed [5, 47].

Clinical trials using COX-2 inhibitors in familial adenomatous polyposis have demonstrated that selective inhibition of COX-2 can alter the development and the progression of cancer [41]. Currently, phase I–II studies using RT in combination with COX-2 inhibitors in, e.g., non-small cell lung, cervical cancer as well as in brain cancer are under way (*DFCI-02024* [Celecoxib in preventing cancer in patients with oral leukoplakia and/or head and neck dysplasia. Phase II pilot study of celecoxib as chemoprevention of HNSCC in patients with oral leukoplakia and/or dysplasia] and *DFCI-03029* [Open label, phase I study of ZD1839 (IRESSA™) plus celecoxib (CELEBREX™) in patients with metastatic, recurrent or refractory head and neck squamous cell carcinoma, with surrogate endpoint biomarker analysis phase: I]).



Figures 1a and 1b. Survival after irradiation in SCC9 (a) and SCC25 (b) cell lines. Cells were irradiated with increasing doses from 1 up to 6 Gy.

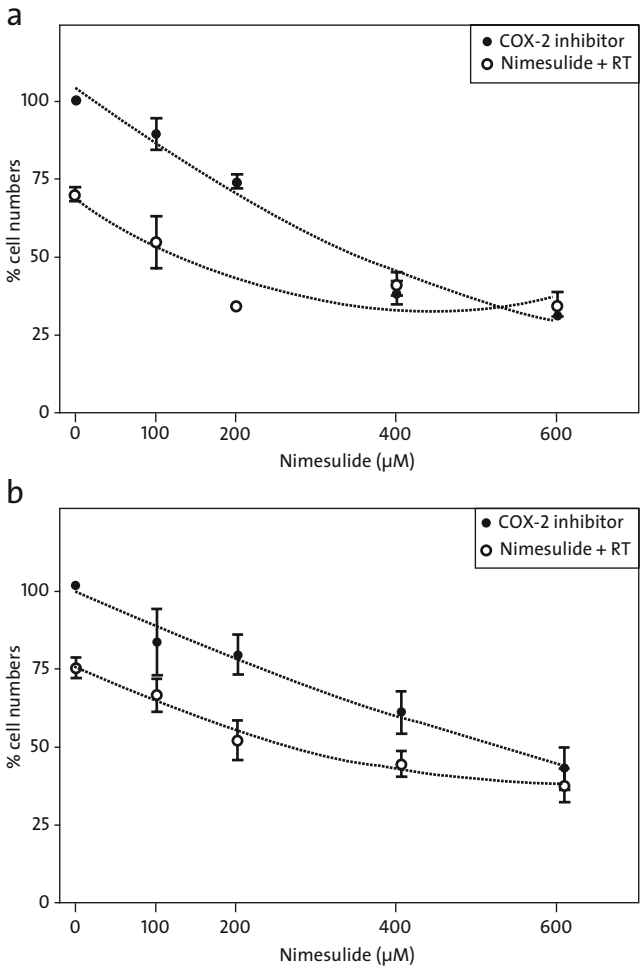
Abbildungen 1a und 1b. Zellproliferation von SCC9 (a) und SCC25 (b) nach Bestrahlung. Die Zellen wurden mit steigenden Dosen von 1 bis zu 6 Gy bestrahlt.

In this work, we investigated whether the COX-2-selective inhibitor nimesulide would enhance the effect of radiation in HNSCC cell lines.

Material and Methods

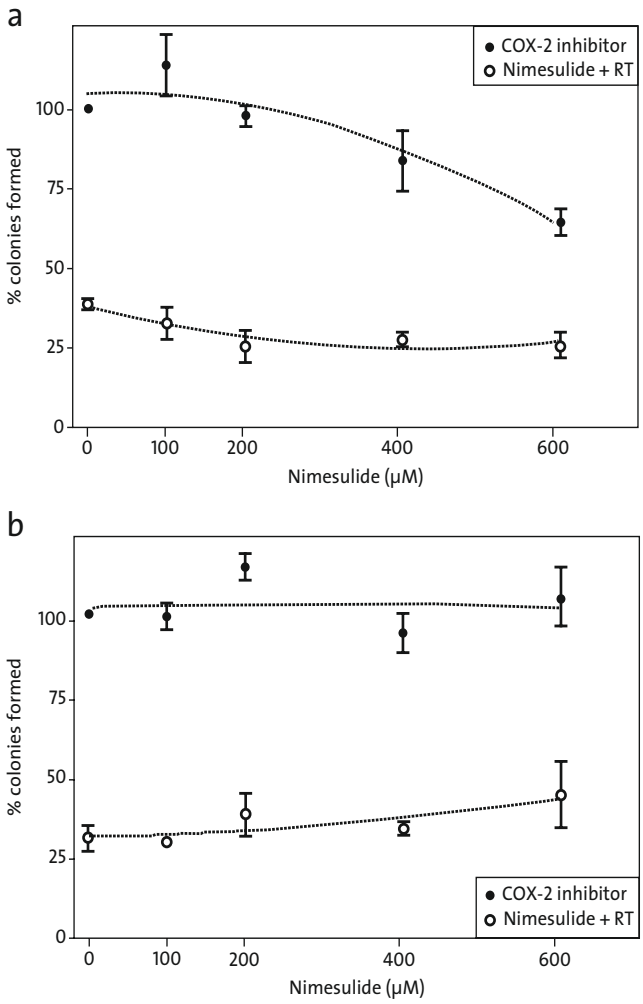
Study Drug

Nimesulide (Helsinn Chemistry, Dublin, Ireland) was provided as a pure substance and dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 1 M and stored at –20 °C.



Figures 2a and 2b. Effects of sequential treatment with nimesulide and/or RT on growth of SCC9 (a) and of SCC25 (b) cells. Cell lines were treated with increasing doses of nimesulide (100–600 µM final) and/or 4 Gy.

Abbildungen 2a und 2b. Effekt der zeitversetzten Behandlung mit Nimesulide und/oder Bestrahlung auf das Wachstum von SCC9 (a) und SCC25 (b). Die Zellen wurden mit steigenden Dosen von Nimesulid (100–600 µM) und/oder 4 Gy behandelt.



Figures 3a and 3b. Long-term effects (clonogenic survival) of sequential treatment with nimesulide in combination with RT on SCC9 (a) and of SCC25 cells (b).

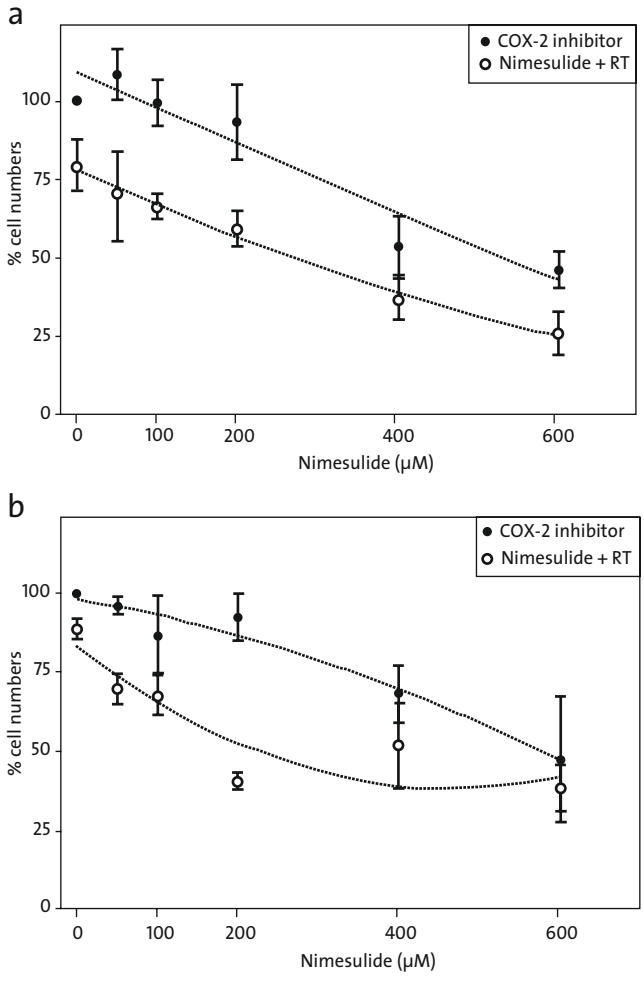
Abbildungen 3a und 3b. Langzeiteffekt auf die Koloniebildungsfähigkeit von SCC9 (a) bzw. SCC25 (b) nach zeitversetzter Behandlung mit Nimesulid in Kombination mit Bestrahlung.

Cell Culture

HNSCC cell lines SCC9 and SCC25 were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown as monolayers in RPMI medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (all reagents from Life Technologies Ltd., Paisley, Scotland, UK). Cell lines were maintained in a humidified 5% CO_2/95% atmosphere at 37 °C. Cells were irradiated using a conventional X-ray source with a dose rate of 1 Gy/0,73 min. Cells numbers were then counted at time points as indicated in the text. For treatment with nimesulide, cells were seeded into 10-cm dishes (5×10^5 cells per dish) and nimesulide was added at increasing concentrations (50–600 µM). Control dishes were treated with an equivalent concentration of DMSO which was given at the highest concentration of nimesulide. For the sequential experiments, cells were irradiated 48 h after drug treatment with a single dose of 4 Gy. For the concomitant experiments, cells were incubated with nimesulide followed immediately by irradiation at 4 Gy. Cells were then trypsinized and counted with the automated CASY® Cell Counting System (Schärfe System GmbH, Reutlingen, Germany).

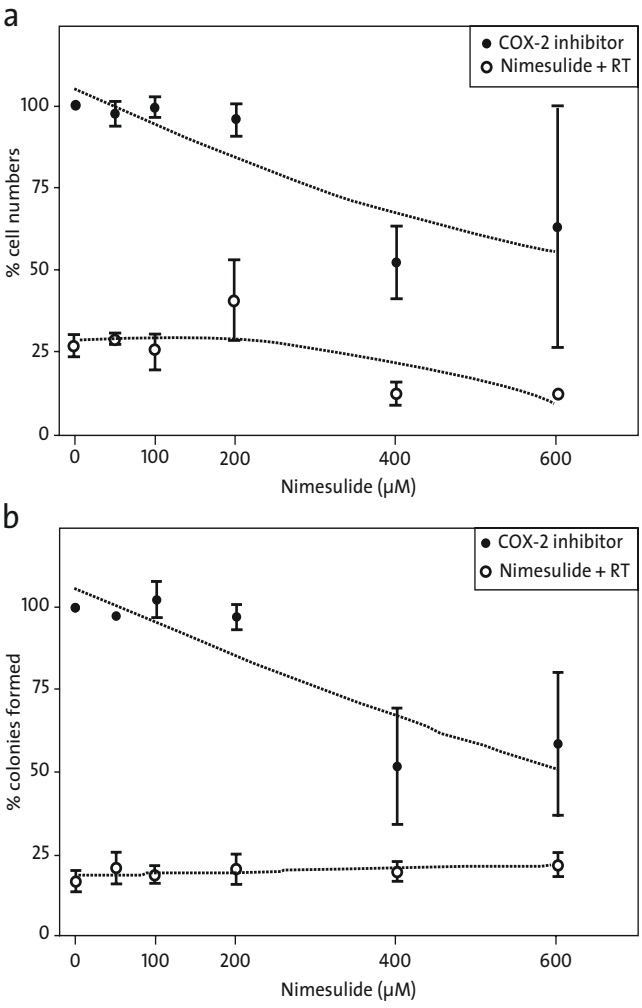
Colony-Forming Assays

For colony-forming assays, 4×10^2 cells per well were plated in six-well plates in drug-free medium. 10 days after treatment,



Figures 4a and 4b. Effects of concomitant treatment with nimesulide in combination with RT on cell growth of SCC9 (a) or SCC25 (b) cells.

Abbildungen 4a und 4b. Kurzzeitergebnisse nach konkomitanter Therapie mit Nimesulid in Kombination mit Bestrahlung von SCC9 (a) und SCC25 (b).



Figures 5a and 5b. Clonogenic survival in SCC9 (a) and SCC25 (b) after concomitant treatment with nimesulide and RT.

Abbildungen 5a und 5b. Klonogenizitätskapazität von SCC9 (a) und SCC25 (b) nach konkomitanter Therapie mit Nimesulid und Bestrahlung.

colonies were fixed with paraformaldehyde and stained with methylene blue. Colonies consisting of > 50 cells were regarded as clonogenic survivors and counted.

Flow Cytometry Analysis

Cell-cycle analysis was performed as follows: at various time points after treatment, SCC25 and SCC9 cells were harvested, washed twice in phosphate-buffered saline (PBS) and fixed in 70% ice-cold ethanol. Immediately before flow cytometry analysis, cells were pelleted, washed and resuspended in PBS containing 50 mg/l propidium iodide and RNase A (Böhringer Mannheim, Mannheim, Germany). After 30-min incubation at room temperature in the dark, fluorescence intensities were measured by flow cytometry (EPICS-XL MCL, Beckman Coulter, Fullerton, CA, USA) and analyzed with Multicycle AV software version 3.0 for Windows (Phoenix Flow Systems, San Diego, CA, USA).

Statistical Analysis

Statistical analysis was performed using SigmaPlot 2002 for Windows version 8.0 (Systat Software Inc., Richmond, CA, USA). Comparisons of means were carried out by Student's unpaired t-test and a p-value < 0.05 was considered statistically significant. Combination effects were quantified using the formula described by Aapro et al. [1]. Error bars represent standard errors of the means (SEM) of the experiments which were repeated three times.

Results

Effect of Irradiation Alone or in Combination with Nimesulide on Cell Survival

For survival experiments, we used two well-described HNSCC cell lines, SCC9 and SCC25. Our group has recently reported high-level expression of COX-2 enzyme in these cells [33]. At first, we determined the effect of irradiation on cell survival in both cell lines. A dose-response curve for survival at different concentrations after RT alone is shown in Figures 1a and 1b. Both cell lines were irradiated with doses of 1, 2, 4, or 6 Gy and cell numbers determined after 48 h. At 4 Gy, cell numbers were reduced to 51.56% (SEM ± 0.76%) in the SCC9 cell line and to 44.08% (SEM ± 0.85%) in the SCC25 cells after 48 h. Therefore, a dose of 4 Gy, which reduced cell numbers by approximately 50% in both cell lines, was chosen for the further experiments to determine radiosensitizing effects of nimesulide on SCC9 and SCC25 cells. After 24 h, irradiation with 4 Gy led to a reduction in cell numbers by 30.32% ± 3.19% (SCC9, Figure 2a) and by 26.15% ± 4.22% (SCC25, Figure 2b). In order to assess combination effects after treatment with RT and with nimesulide, a dose of 4 Gy was chosen.

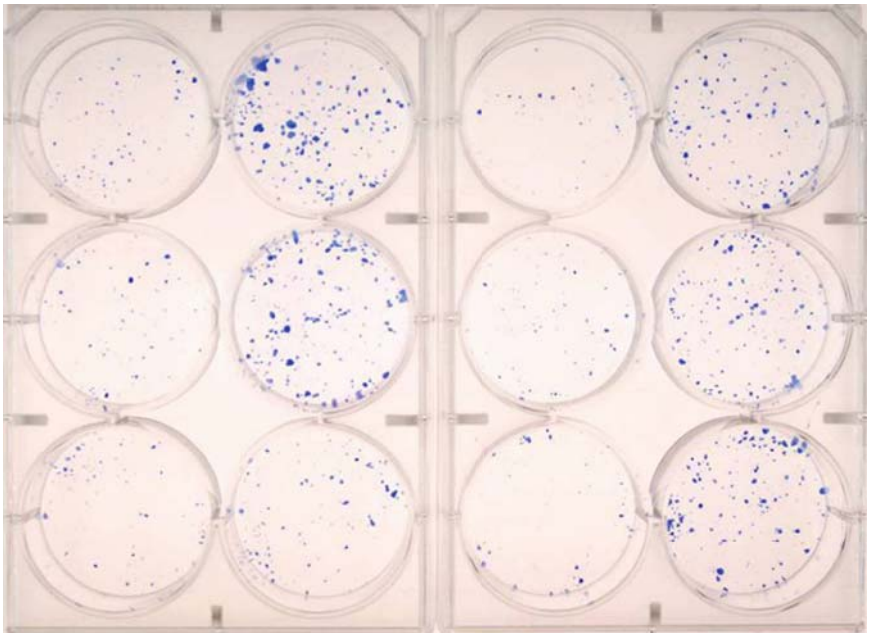


Figure 6. Representative photomicrograph of a clonogenic assay of SCC25 cells after concomitant treatment.

Abbildung 6. Repräsentatives Foto eines klonogenen Assays von SCC25-Zellen nach konkomitanter Behandlung.

Next, we performed an analysis of dose-response effects of nimesulide on survival of SCC9 and SCC25 cells with or without irradiation (Figure 2). Cell survival decreased significantly in both cell lines after treatment with increasing concentrations of nimesulide. At the highest concentration of nimesulide, survival was 31.41% (SEM ± 1.60%) for SCC9 cells and 42.50% (SEM ± 7.97%) for SCC25 cells. Dose-dependent growth-inhibitory effects were similar to the data published by another group for example in human gastric adenocarcinoma cells [22]. We used the formula described by Aapro et al. [1] in order to determine the combination effects of nimesulide and RT. As is immediately evident from a visual inspection of the survival curves alone, we could not find any evidence for a beneficial effect of a combination of nimesulide with RT. Taken together, analysis of the combined effects showed no evidence for an additive effect in the combined treatment groups. In fact, at higher concentrations of nimesulide alone, survival was almost identical to the RT plus nimesulide group.

Short-term survival assays, such as measurements of cell proliferation after 3 days, may not reflect the clinically more relevant clonogenic potential of cells treated by chemo- and/or radiotherapy. We therefore performed colony formation assays with both cell lines after treatment with RT in the presence or absence of nimesulide. These experiments are shown in Figures 3a and 3b. In both cell lines, treatment with nimesulide alone did not significantly affect clonogenic survival. RT alone reduced clonogenic survival by 61.72% (SEM ± 3.70%) in SCC9 cells and by 70.83% (SEM ± 3.60%) in SCC25 cells. Furthermore, we could not find any significant decrease of colony-forming ability when cells were treated with nimesulide in combination with RT.

Effect of Irradiation Alone or Concomitantly with Nimesulide on Cell Survival

In the experiments described above, treatments were performed sequentially. Since, in patients, both treatments are usually given concomitantly, we performed cell survival assays investigating the effects of a concomitant therapy of nimesulide with RT (Figures 4a and 4b). Again, no synergism could be demonstrated and at higher concentrations of nimesulide the effects turned out to be inhibitory in both cell lines.

In agreement with the results of the sequential experiments shown above, RT alone led to a significant reduction of colonies formed, while nimesulide, even at concentrations leading to clear growth-inhibitory effects in proliferation experiments, alone or in combination with RT was inhibitory (Figures 5a, 5b and 6).

Effect of Nimesulide on Cell-Cycle Distribution

Since the effects of RT on cell survival are cell-cycle-dependent, we performed cell-cycle analyses of nimesulide-treated cells. Using flow cytometry analysis, we assessed the influence of nimesulide on cell-cycle distribution of SCC9 and SCC25 after 24 h, 48 h, and 72 h. In contrast to the findings of Li et al. in gastric carcinoma cells [22], treatment with nimesulide between 50 μM up to 600 μM did not significantly affect distribution of HNSCC cells within G1, S or G2 phase compared to control-treated cells (Figures 7a and 7b).

Discussion

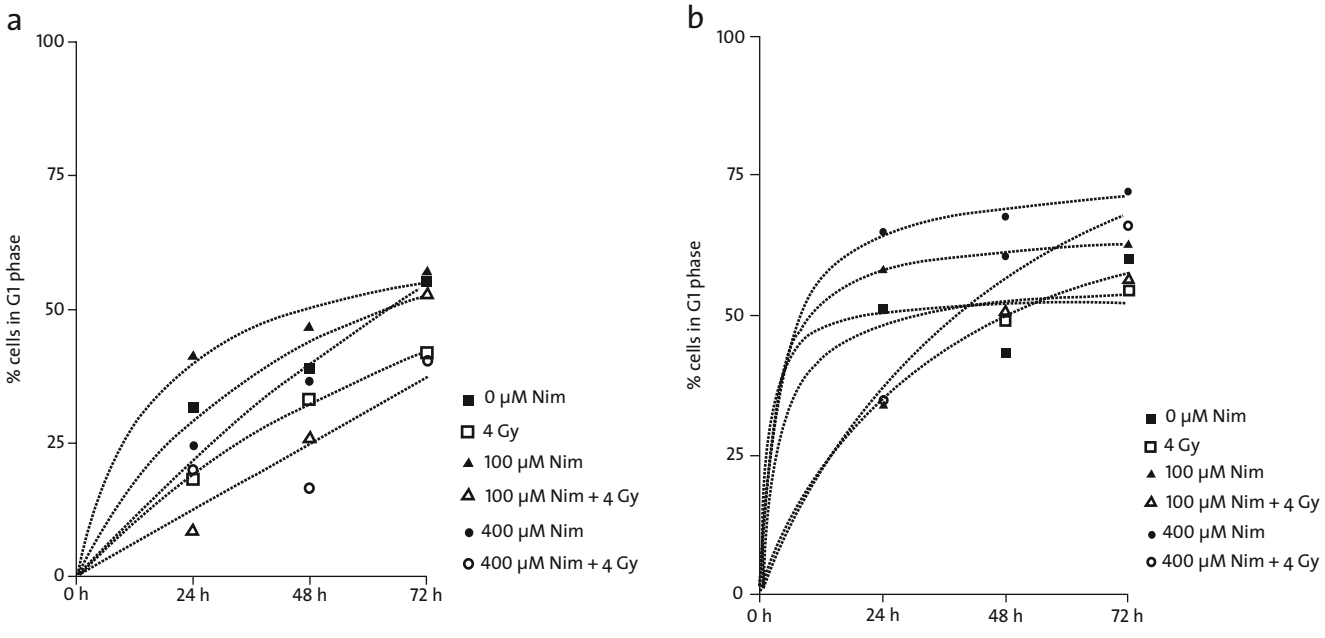
Despite recent advances in anticancer strategies, the median survival time for the majority of head-and-neck cancer patients still remains very poor due to the high incidence of local recurrences as well as metastases [42], but also to a lesser extent due to the occurrence of secondary primaries [8]. There is evidence that preoperative neoadjuvant simultaneous radiochemotherapy seems to prospectively increase a long-term tumor-free survival [12, 19]. Aiming for further improvement there is a demand of identifying new molecular targets and novel combined treatment modalities and chemopreventive agents [10].

One of such novel molecular anticancer target proteins along with others (e.g., [38]) might be the COX enzyme splice variant 2. Some of the so-called selective COX-2 inhibitors have been found to improve tumor response to RT in a number of rodent tumor and human tumor xenografts [5]. In this study, we combined the selective COX-2 inhibitor nimesulide with irradiation in order to modulate radiosensitivity of SCC9 and SCC25, two head-and-neck cancer cell lines.

Nimesulide alone exhibited a cytotoxic effect in both cell lines in concentrations which are pharmacologically relevant in humans.

In our experiments, the application of nimesulide in combination with RT did not result in radiosensitization of squamous carcinoma cells as observed by other groups when combining RT with lovastatin, a member of the drug class of statins [16]. In fact, our data even indicated subadditive effects of this combination. This was confirmed both by short-term proliferation as well as in colony-forming assays, irrespective of whether these treatments were applied in a sequential manner or concomitantly. Interestingly, we found no significant alteration in cell-cycle distribution after treatment with nimesulide as assessed by flow cytometry analysis. These results are comparable to those made by Bartkowiak et al. who also could not find a radiation response-modifying effect of imatinib, a tyrosine kinase inhibitor, but even revealed a radioprotective effect of this substance. Furthermore, there were only minor cell-cycle alterations in the presence of imatinib [2].

The outcomes of our experiments using nimesulide are in contrast to the results published by other groups using chemically different COX-2 inhibitors. For example, Petersen et al. could show that treatment of human glioblastoma



Figures 7a and 7b. Flow cytometry analyses: distribution of cells in G1 or any other phase comparing the control with the nimesulide-treated SCC9 (a) and SCC25 (b) cells.

Abbildungen 7a und 7b. Durchflusszytometrie: Verteilung der Zellen in der G1- oder einer anderen Phase im Vergleich Kontrollzellen versus nimesulidbehandelte SCC9- (a) und SCC25-Zellen (b).

cells with SC-236, a selective COX-2 inhibitor, caused an increase in radiosensitivity [34]. Pyo et al. found an enhancement of radiosensitivity by NS-398, an experimental COX-2 inhibitor, in rat intestinal epithelial cells which were stably transfected with COX-2 cDNA but not cells which were transfected with COX-2 cDNA in the antisense orientation. Furthermore, they showed that NCI-H460 human lung cancer cells which express COX-2 were radiosensitized by NS-398 but not HCT-116 human colon cancer cells which do not express COX-2 [36], thus indicating that COX-2 is crucially involved in pathways affecting radiation-enhancing effects. Liu et al. found a radiosensitizing effect of the selective COX-2 inhibitor celecoxib in breast tumor cell line MCa-35 but not in lung carcinoma cell line A549, although both cell lines express COX-2 [25]. Both cell lines used for our study, SCC25 and SCC9, express COX-2 as has recently been demonstrated by our group [33], but neither of them is radiosensitized by the COX-2 inhibitor nimesulide. Taken together, these observations may indicate that some effects of selective COX-2 inhibitors are cell-type- or tissue-type-specific. In addition, it is conceivable that different selective COX-2 inhibitors have different effects on radiosensitivity as well. In contrast to the effects of COX-2 inhibitors with RT, chemopreventive effects of COX-2 inhibitors have been more thoroughly investigated as well as their capability to suppress metastatic spread [3, 6, 24, 31, 35, 40].

Taken together, the results of our experiments clearly show that nimesulide affects proliferation but has no impact on clonogenic survival. By contrast, irradiation is able to affect proliferation as well as clonogenic survival [28]. As has been demonstrated for the influence of the anti-apoptotic protein Bcl-2 on cellular survival, inhibition of proliferation does not necessarily have to be accompanied by a reduction in long-term clonogenic survival. On the other hand, effects of irradiation on proliferation, but not on clonogenic survival, are reduced by the anti-apoptotic protein Bcl-2 [21]. In contrast to Bcl-2, another anti-apoptotic Bcl-2 family member – Mcl-1 – is able to inhibit both early cell death and clonogenic survival [39] in response to irradiation. Results of other groups and our own data presented here therefore show that caution has to be exerted when trying to extrapolate results of short-term cytotoxicity assays and to predict, thereof, the potential long-term clinical tumor response even when using the same class of compounds.

Acknowledgment

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