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Preclinical report

Effects of tamoxifen on human squamous cell carcinoma lines of the head and neck

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Tamoxifen (TAM) is a well-tolerated compound in the treatment of breast cancer and is primarily considered to act by competition with estrogen receptors (ER). Here we investigated the *in vitro* efficacy and potentially underlying mechanisms of TAM in established cell lines of squamous cell carcinomas of the head and neck (SCCHN). Using proliferation and apoptosis assays the antitumor activity of TAM in five SCCHN and the breast carcinoma line MCF-7 (positive control) was determined. MCF-7 was more sensitive to low-dose TAM (below 1 μM), whereas SCCHN showed significant growth inhibition at higher TAM concentrations (5–10 μM). Growth curve analysis and apoptosis assays were indicative for a cytostatic effect of low-dose TAM and high-dose TAM led to cell loss by apoptosis in sensitive SCCHN. In order to further characterize the observed antitumor effects we determined the amount of steroid hormone receptors with the dextran-coated charcoal method and immunocytochemistry. In addition, production of transforming growth factor (TGF-)-α, -β1 and -β2 was measured by ELISA, and protein kinase C (PKC) activity was assessed with a radioligand assay. Except MCF-7, none of the SCCHN lines was positive for ER. TAM caused decreased TGF-α and increased TGF-β levels in MCF-7, but not in SCCHN supernatants. Furthermore, the antiestrogen reduced PKC activity in MCF-7, but not in SCCHN. In the present *in vitro* system, the observed antitumor activity of high-dose TAM in SCCHN cannot be explained by estrogen antagonism, alterations of TGF-α/β levels or decreased PKC activity. [© 2002 Lippincott Williams & Wilkins.]

Key words: Apoptosis, growth factors, head and neck cancer, protein kinase C, tamoxifen.

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Introduction

Tamoxifen (TAM) is widely used in the adjuvant treatment of breast cancer. Since antitumor activity has been predominantly observed in patients with estrogen receptor (ER)+ tumors, it appears that major effects of TAM are mediated through competitive antagonism of estrogen at its receptor.[1,2] However, subgroups of ER– breast cancer also respond to TAM treatment and TAM alone or in combination with chemotherapy is believed to bear useful antineoplastic activity in other solid tumors without apparent estrogen dependency, in particular malignant melanoma.[3–12]

Potential antitumor mechanisms underlying the hormone-unrelated effects of TAM are a continuing issue of research and discussion. In view of the well-established concept that manifestation of the malignant phenotype requires tumor autonomy which is largely provided by peptide growth factors,[13] the quality of TAM to modulate these positive and negative regulators of tumor cell proliferation, differentiation and apoptosis has attracted considerable interest. In breast cancer TAM is able to exert growth inhibition via suppression of auto-stimulatory TGF-α production[14,15] and by increasing the secretion of TGF-β, which is known to act antiproliferatively on a number of epithelial malignancies.[16] In addition, TAM has been shown to stimulate the secretion of TGF-β in evidential ER– fibroblasts, possibly contributing to its antineoplastic activity *in vivo*.[17,18] Furthermore, TAM interferes with the activity of protein kinase C (PKC), an enzyme playing a pivotal role in signal transduction pathways involved in the control of cell proliferation and differentiation.[19,20] In ER– human malignant glioma

cells, TAM-mediated PKC inactivation was accompanied by increased numbers of apoptotic events.[21]

In the present paper, we compared the effects of TAM on tumor cell proliferation and induction of apoptosis in five established squamous cell carcinomas of the head and neck (SCCHN) cell lines and the ER+ breast carcinoma line MCF-7. The substantial antitumor activity of high-dose TAM in SCCHN prompted us to investigate commonly suggested modes of action, including hormone status, TGF-α, -β1 and -β2 protein secretion, as well as PKC activity.

Materials and methods

Cell lines and cell culture

SCCHN cell lines UM-SCC 11B, UM-SCC 14C and UM-SCC 22B were kindly provided by TE Carey (University of Michigan, Ann Arbor, MI). UM-SCC 11B was established from a larynx carcinoma, UM-SCC 14C from the local recurrence of a carcinoma of the floor of mouth and UM-SCC 22B originated from the lymph node metastasis of a hypopharynx carcinoma.[22] 8029 NA was derived from the neck metastasis of a larynx carcinoma and cell line 8029 DDP represents a recloned subpopulation which has been made 5-fold resistant to the antineoplastic agent cisplatin by incremental drug exposure.[23,24] The ER+ human breast cancer cell line MCF-7[25] served as positive control. All cells were grown as monolayers in plastic culture flasks (Becton Dickinson, Heidelberg, Germany) under standard conditions (37°C, 5% CO_2, fully humidified atmosphere) using modified Eagle's medium supplemented with 10% heat-inactivated single-batch fetal calf serum (both Gibco, Eggenstein, Germany), 2 mM L-glutamine, 50 μg/ml streptomycin and 50 IU/ml penicillin (all ICN, Meckenheim, Germany) as described previously.[26] The growth medium for MCF-7 cells was additionally supplemented with 2% non-essential amino acids (Gibco). To transfer or passage cell lines, confluent monolayers were washed with phosphate-buffered saline (PBS) and detached with 0.05% trypsin/0.02% EDTA solution (Boehringer, Mannheim, Germany). All cultures were routinely tested to be free of mycoplasma contamination (Enzym-Immunoassay; Boehringer). The entire study was performed within a maximum of 20 cell passages.

Reagents

TAM-citrate (M_r 563.65), a friendly gift from Zeneca (Plankstadt, Germany), was dissolved in ethanol at 1000 times the highest concentration employed *in vitro* and stored as stock solution in aliquots at −20°C until used. Final concentrations between 0.1 and 10 μM TAM were obtained by corresponding dilution of the stock compound with complete growth medium. TAM concentrations applied *in vitro* were orientated on clinically achieved levels of TAM in serum and tumor tissue under standard treatment (0.1–1 μM) as well as high-dose therapy (1–10 μM).[27–29] In control experiments, final growth medium concentrations of the solvent ethanol (below 0.1%) did not affect viability, growth behavior or microscopic appearance of tumor cells.

In vitro growth inhibition/cytotoxicity assay

Antitumor activity of TAM was determined with the MTT test (Sigma, Munich, Germany). The colorimetric assay is based on the reduction of a non-toxic water-soluble yellow tetrazolium salt to a purple-colored water-insoluble formazan precipitate by the reductive capacity of cytoplasmatic and mitochondrial dehydrogenases present only in living metabolically active cells. On day 0, exponentially growing tumor cells were harvested from culture flasks and resuspended in 96-well flat-bottom microtiter plates (Becton Dickinson, Heidelberg, Germany) at 6×10^3 cells/200 μl complete growth medium/well. On day 3, culture medium of consolidated tumor cell monolayers was replaced by fresh medium containing 0.1, 0.5, 1.0, 5.0 or 10.0 μM TAM and controls received medium alone. Since untreated cells almost reached confluency on day 6, the MTT assay was performed after incubation with TAM for 24 and 72 h. For the 72-h incubation interval either single drug application on day 3 or multiple drug applications on day 3, 4 and 5 (total dosages: 0.3, 1.5, 3.0, 15.0 or 30.0 μM TAM) were administered. MTT solution (50 μl) was added to each well and left to react for 4 h. After complete dissolving of formazan precipitates in 150 μl dimethylsulfoxide (Sigma), absorbance was measured at 570 nm in a microplate reader (Ear 400 ATX; SLT-Labinstruments Crailsheim, Germany). Wells with all components of the mixtures except cells served as blanks. In previous investigations, this experimental procedure has been identified to produce a good correlation between absorbance and the number of viable tumor cells.[26,30] Therefore, tumor cell survival as percentage of the control (fractional absorbance) was calculated according to the formula: fractional absorbance (cell survival) = (absorbance test − absorbance blank)/(absorbance control − absorbance blank) × 100. MTT assays were

performed as 6-fold replicates in three independent experiments and values were tested in a paired *t*-test for significance.

In order to validate actual cell loss we additionally performed cell count experiments using Trypan blue exclusion. On day 0, tumor lines were seeded at 8×10^4 cells/2.7 ml medium/well in 12-well flat-bottom microtiter plates (Becton Dickinson). On day 3, monolayers were treated for 24 or 72 h with 1 and 10 μM TAM, respectively, and the number of unstained cells was counted. Three independent experiments were performed in duplicate, and results are specified as mean cell number $\pm$ SEM.

Analysis of apoptosis

Similar as described previously,[31] apoptosis was determined with both ELISA (Cell Death Detection Plus; Boehringer) and *in situ* nick end-labeling (TUNEL, In Situ Cell Death Detection Kit; Boehringer) 24 and 72 h after addition of TAM. Controls remained untreated. The ELISA is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, which allows specific detection of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. ELISA results are shown as intensification factor (IR), defined as: IR=extinction in experimental probe/extinction in control probe referred to 1×10^6 cells. For the TUNEL assay, 6×10^3 tumor cells were seeded on 16-well chamber slides (Nunc, Naperville, IL), incubated under standard conditions for 72 h, and exposed to 0, 1 or 10 μM TAM for another 24 and 72 h, respectively. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After further washing with 2% BSA, slides were treated with acidic ethanol (10 ml absolute ethanol+20 μl 37% HCl) for 20 min and washed repeatedly with PBS. TUNEL reaction was performed in a moist chamber through incubation with a mixture (total volume 50 μl) of 0.3 nmol fluorescein–dUTP, 3 nmol dATP, 2 μl 25 mM $CoCl_2$, 25 U terminal deoxynucleotidyl transferase (TdT) and TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate) at 37°C. Slides were rinsed with PBS, incubated with sheep serum (Sigma, St Louis, MO) for 45 min at 37°C and sheep–fluorescein antibody $F(ab')_2$ fragments conjugated with horseradish peroxidase (mixed 1:1 with sheep serum) for 30 min at 37°C. Finally, the slide mounted cells were incubated with diaminobenzidine (DAB) and counter-stained with Mayer's hemalum. For positive controls, cell monolayers were treated with DNase I (10 μg/ml, 10 min at room temperature) to induce DNA strand breaks. In negative controls, TdT was omitted.

ER assays

Dextran-coated charcoal (DCC) method and Scatchard plot. The ER content was determined with a multiple-point DCC assay. Tumor cells incubated under standard conditions were harvested mechanically during logarithmic growth on day 6 after subcultering and approximately 500 mg of each cell line were immediately stored at −80°C. All further processing of the material was performed at 0–3°C. Cells were powdered in a tissue pulverizer (Braun, Melsungen, Germany), homogenized with three 10-s bursts in a homogenizer, and resuspended in buffer containing 0.01 mol/l K_2HPO_4/KH_2PO_4 (pH 7.5), 0.0015 mol/l K_2EDTA, 0.003 mol/l, NaN_3, 10% (v/v) glycerin and 0.01 mol/l monothioglycerin. Homogenates were centrifuged at 104 000 *g* at 3°C for 45 min. Resulting cytosol fractions were screened for ER activity with a competitive protein-binding assay in which DCC (1% Norit A and 0.05% Dextran T500 in extraction buffer) is used to separate unbound [^{3}H]estradiol-17β (Gestagen; Amersham, Germany) from unspecific protein associated radioactivity. Multiple concentrations of [^{3}H]estradiol-17β were utilized for the construction of Scatchard plots of the binding data.[32] The first-order dissociation rate constant was determined from the slope of plot bound/free versus bound steroid hormone. Protein content of the probes was determined with the BioRad Protein Microassay (BioRad, Munich, Germany) and the amount of receptor is expressed as fmol/mg protein. Values above 10 fmol [^{3}H]estradiol-17β binding/mg cytoplasmic protein were considered positive. MCF-7 cells served as positive control.

Immunocytochemistry. For immunocytochemical ER detection we used the ER-ICA monoclonal antibody kit as described by the manufacturer (Abbott Laboratories, Wiesbaden, Germany). Briefly, 4–8-μm cryosections of cell pellets were placed on tissue adhesive glass slides, fixed for 10 min in 3.7% formaldehyde/PBS, immersed for 10 min in PBS, fixed in ice-cold methanol for 4 min and ice-cold acetone for 2 min, and again immersed in PBS. Following 15 min of incubation with 2% normal goat serum in PBS in order to reduce non-specific binding, slides were incubated for 30 min with the primary rat monoclonal antibodies H222 for ER. After

washing with PBS, slides were incubated for 30 min with goat anti-rat bridging antibody and for 30 min with horseradish peroxidase–anti-horseradish peroxidase. Subsequently, slides were incubated for 6 min with freshly-mixed chromogen (DAB) and hydrogen peroxide in PBS. Sections were counterstained with 1% Harris hematoxylin prior to dehydration and mounting in xylene.

Two independent observers (HB and TKH) assessed the slides for the percentage of stained cells. Cell lines were classified ER+ when more than 20% tumor cells stained positive. Negative controls were exposed to normal rat serum instead of specific monoclonal anti-ER antibodies. Again, MCF-7 cells served as positive control.

TGF-α and -β1/2 assays

After seeding 8×10^4 cells/2.7 ml medium/well in 12-well flat-bottom plates (Falcon) on day 0, fresh medium containing no, or 1 respectively 10 μM, TAM was employed on day 3. ELISAs for TGF-α, -β1 and -β2 in tumor cell culture supernatants were performed after 24 and 72 h. Each growth factor was assayed in duplicate in three independent experiments. Concentrations are specified as mean values and the SEM was generally below 15%.

TGF-α protein was determined with a quantitative ELISA (Calbiochem, Darmstadt, Germany) utilizing affinity-purified goat polyclonal antibody specific for mammalian TGF-α, and has been described elsewhere.[30] The minimum concentration of TGF-α which can be detected with this method was 10 pg/ml.

To measure total TGF-β, we transformed latent TGF-β into its immunoreactive form by transient acidification of supernatants prior to the assay (R & D, Wiesbaden, Germany). Supernatants were mixed 5:1 with 1 N hydrochloric acid at room temperature and after 10 min neutralization was obtained with 1.2 N NaOH/0.5 M HEPES (pH 7.2–7.6). Corresponding control aliquots received premixed acid, base and HEPES buffer to ensure isoionic conditions. Both TFG-β assays employ the same quantitative sandwich enzyme immunoassay technique as described for TGF-α, i.e. TGF-β1/β2-specific antibodies pre-coated on microplates bind the respective peptides and after exposure to specific polyclonal detector antibody which is linked to horseradish peroxidase-conjugated streptavidin catalyses the conversion of chromogenic tetra-methylbenzidine. Color intensity was measured with a multireader spectrophotometer using dual wavelengths at 450 and 570 nm (Ear 400 ATX). The detection limit was 2 pg/ml for TGF-β1 and 7 pg/ml for TGF-β2.

PKC activity

Because of their distinct responsiveness to TAM, cell lines MCF-7, UM-SCC 11B and UM-SCC 22B were selected for the determination of PKC activity. After subcultering, cells were grown under standard conditions for 72 h, exposed to 0, 1 or 10 μM TAM for 1, 24, and 72 h, respectively, and harvested mechanically. After washing with PBS, 1×10^7 tumor cells were lysed in 0.5% Triton X-100, and homogenized in buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA and 5 mM EGTA by 5-fold sonication for 10 s on ice. Samples were applied to DEAE–cellulose columns (BioRad), which were washed with 2.5 ml buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA and 2 mM PMSF, and PKC was eluted with the same buffer containing 100 mM NaCl. PKC activity was determined by measuring the Ca^{2+}- and phospholipid-dependent transfer of ^{32}P from [^{32}P]ATP to histone III-S. Both in the absence and presence of 0.75 mM $CaCl_2$, 12 μg phosphatidylserine and 1.6 μg of 1,2-diolein, 100 μl of the eluate was added to 150 μl of a solution containing 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate and 25 μg histone III-S. The transfer reaction was started at room temperature by addition of 50 μM ATP containing 3.3 μCi [^{32}P]ATP per assay, and terminated after 30 min through histone precipitation with ice-cold 25% trichloroacetic acid and 0.1% bovine serum albumin. Precipitates were collected on 0.45 μm nitrocellulose filters (GF/A; Whatman, Maidstone, UK) and analyzed in a liquid scintillation counter. PKC activity was determined from the amount of radioactivity incorporated into histone H1.

Results

Growth inhibition

TAM led to time- and dose-dependent growth inhibition in all cell lines. After 24 h, MTT assays revealed no significant effect for 1 μM TAM, whereas 10 μM TAM led to growth inhibition of 20% in most SCCHN lines (data not shown). After single TAM application for 72 h, breast cancer line MCF-7 appeared to be the most sensitive tumor to low-dose TAM (Table 1 and Figure 1). Already 0.1 μM TAM caused significant growth inhibition of 19%, which

was further enhanced to 40% by increasing the concentration to 10 μM. In contrast, most SCCHN lines appeared to be slightly growth stimulated by low-dose TAM (0.1–1 μM), but were significantly growth inhibited by high-dose treatment. Using 10 μM TAM, UM-SCC 22 B and UM-SCC 11B were the most sensitive SCCHN lines showing growth inhibition up to 60% (Table 1 and Figure 1). Replacement of TAM-containing medium by normal medium allowed all cell lines to recover from TAM-induced growth inhibition (not shown). Dose intensification via administration of 10 μM TAM on 3 consecutive days regularly led to tumor eradication in both SCCHN and MCF-7 cell lines.

To substantiate the cytotoxic effect of TAM in the course of growth inhibition, cell count experiments were carried out. As exemplary shown for cell lines UM-SCC 11B and UM-SCC 22B, 10 μM TAM led to progressive cell loss (Figure 2) and these data correspond to the decrease in metabolic activity of cells as determined with the MTT assay (Figure 1).

For identification of apoptotic cell death, two assays were employed. Similarly to cell count and MTT assays, 24 h TAM incubation did not markedly increase the apoptosis rate (not shown). Incubation with TAM for 72 h, however, showed that low-dose TAM (1 μM) was effective for the induction of apoptosis in MCF-7 cells, but not in SCCHN cell lines (Figure 3). In the 72-h setting, high-dose TAM (10 μM) led to considerable apoptosis in cell lines UM-SCC 11B, UM-SCC 22B and, to minor extent, UM-SCC 14C (Figures 3 and 4).

Table 1. MTT assay after 72 h TAM exposure

Cell line	0 μM	0.1 μM	0.5 μM	1 μM	5 μM	10 μM
UM-SCC 11B	100 (100)	105 (107)	103 (101)	100 (100)	90 (76)[a]	43[a] (0)[a]
UM-SCC 14C	100 (100)	104 (101)	106 (104)	101 (99)	97 (86)	69[a] (0)[a]
UM-SCC 22B	100 (100)	108 (103)	108 (106)	98 (87)	84 (47)[a]	40[a] (0)[a]
8029 NA	100 (100)	96 (101)	101 (100)	92 (101)	90 (85)	84 (0)[a]
8029 DDP	100 (100)	99 (99)	94 (93)	94 (91)	87 (98)	85 (0)[a]
MCF-7	100 (100)	81[a] (83)[a]	79[a] (76)[a]	75[a] (72)[a]	69[a] (68)[a]	60[a] (0)[a]

Tumor cell survival as mean percentage of the control (fractional absorbance) after single-dose (0.1, 0.5, 1, 5 or 10 μM on day 3) and multiple-dose (0.1, 0.5, 1, 5 or 10 μM on day 3, 4 and 5; values in brackets) TAM application. MTT assays were performed as 6-fold replicates in three independent experiments and values (SEM $< 15\%$) were tested in a paired *t*-test for significance ([a]$p \leqslant 0.05$).

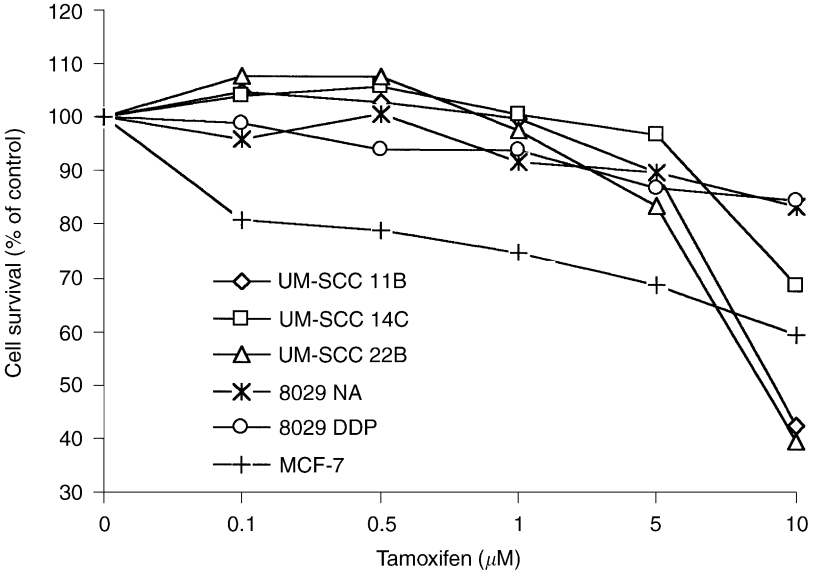

Figure 1. MTT assay after 72-h TAM exposure. Dose–response curves of SCCHN and MCF-7 cell lines showing tumor cell survival as percentage of the control (fractional absorbance) after single-dose (0.1, 0.5, 1, 5 and 10 μM on day 3) TAM application. MTT assays were performed as 6-fold replicates in three independent experiments and the data are specified as mean values (SEM $< 15\%$).

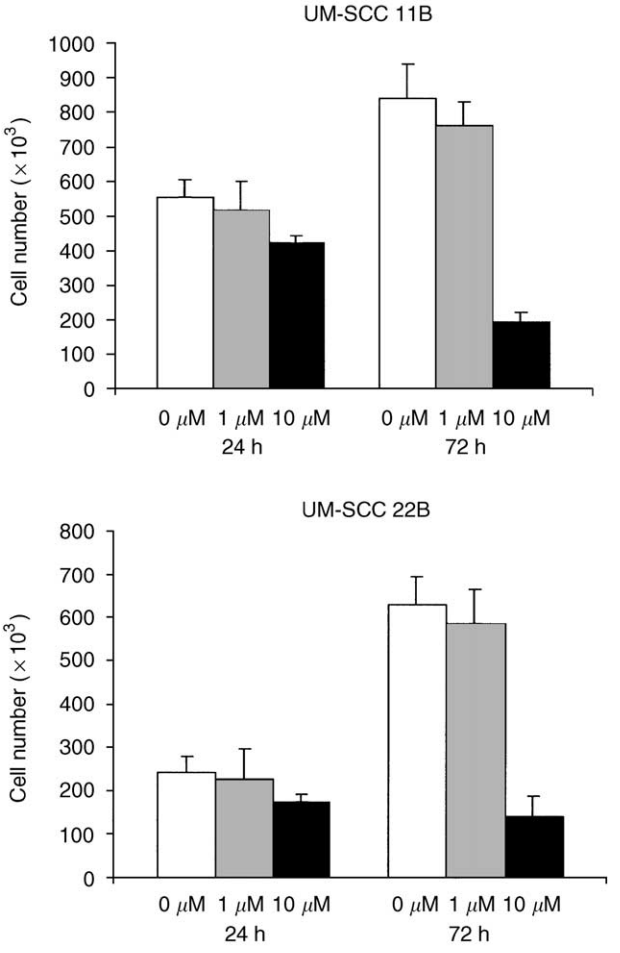

Figure 2. Cell numbers in the TAM-responsive SCCHN cell lines UM-SCC 11B and UM-SCC 22B after treatment with 1 or 10 μM TAM for 24 and 72 h, respectively. Three independent experiments were performed in duplicate, and results are specified as mean cell number $\pm$ SEM.

Steroid hormone receptors

Determined with the DCC method, the ER protein content in SCCHN cell lines was always below 4 fmol/mg protein (Table 2). Therefore, none of the SCCHN lines was considered ER+ in the DCC assay. In contrast, MCF-7 cells were positive for ER, exhibiting 70 fmol ER/mg protein. By immunocytochemistry, MCF-7 cells were again found to be ER+ (60.5% cells stained), whereas all SCCHN lines were absolutely negative for ER expression (Table 2).

TGF-α, -β_1 and -β_2

With the ELISA employed, no TGF-α was detectable in complete culture medium (control). Of the SCCHN, only two cell lines (UM-SCC 11B and 8029 NA) showed detectable amounts of TGF-α in the cell culture supernatant. In both cases incubation with 1 or 10 μM TAM did not cause relevant modifications of the TGF-α level in culture media after 24 and 72 h, respectively (Table 3). Supernatants of untreated MCF-7 cells contained 60 pg TGF-α/ml, and exposure to 1 and 10 μM TAM halved TGF-α levels after 72 h (Table 3). TGF-β1 (760 pg/ml) and TGF-β2 (100 pg/ml) were already found in complete culture medium supplemented with single batch fetal calf serum (control). Clearly increased TGF-β1/2 levels were detected in culture growth media of most untreated tumor cell lines. Highest TGF-β1 levels were found in supernatants of UM-SCC 11B (1388 pg/ml), 8029 DDP (1210 pg/ml) and 8029 NA (965 pg/ml), whereas TGF-β2 was particularly increased in 8029 NA (1055 pg/ml) and 8029 DDP (930 pg/ml). In SCCHN, exposure to 1 or 10 μM TAM did not change TGF-β1 levels after 24 h and led to either unchanged or decreased levels after 72 h (Table 3). Only supernatants of MCF-7 cells revealed a moderate increase in TGF-β1 levels upon 72 h exposure to 1 (840 versus 770 pg/ml) and 10 μM TAM (830 versus 770 pg/ml). TGF-β2 levels remained largely unaltered in all cell lines.

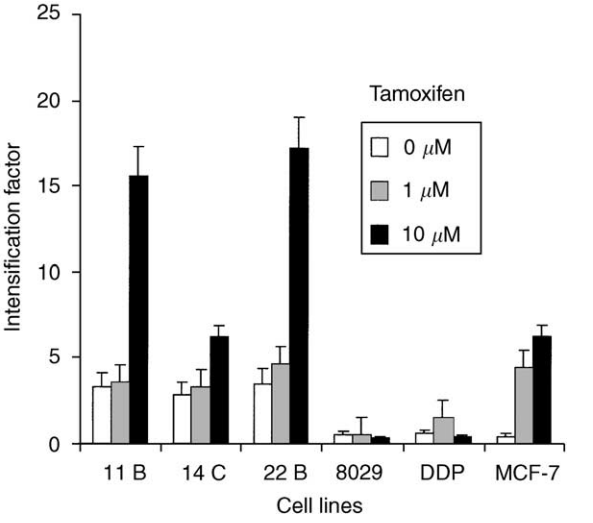

Figure 3. Detection of apoptosis by ELISA. Tumor cells were treated with 0, 1 or 10 μM TAM for 72 h. The intensification factor is defined as extinction of experimental group/extinction of control group referred to 1×10^6 cells. Results of three independent experiments are specified as mean intensification factor $\pm$ SEM.

PKC activity

Only in MCF-7 cells, 10 μM TAM led to a decrease in PKC activity after 1 h incubation, whereas this

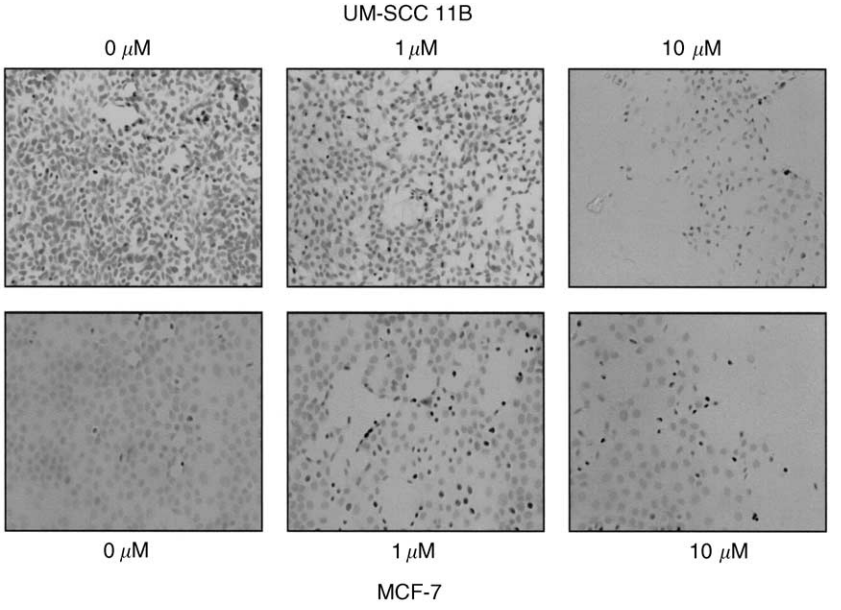

Figure 4. Detection of apoptosis by TUNEL. Tumor cells were treated with 0, 1 or 10 μM TAM for 72 h. Examples shown are preparations of the TAM-responsive SCCHN cell line UM-SCC 11B in comparison to MCF-7 cells (magnification $\times$ 62.5).

Table 2. Determination of ER status

Cell line	Biochemical method (fmol/mg protein)	Immunocyto-chemistry (% positive cells)
UM-SCC 11B	4 (0.4)	0
UM-SCC 14C	1 (ND)	0
UM-SCC 22B	0 (ND)	0
8029 NA	0 (ND)	0
8029 DDP	1 (0.5)	0
MCF-7	70 (0.38)	60.5

Biochemical DCC method with specific binding capacity (fmol/mg protein) and dissociation constant (nM; in brackets; ND, not done), and immunocytochemistry (percentage of stained cells). Cut-off values for ER positivity were $>$ 10 fmol/mg protein and $>$ 20% stained cells, respectively.

effect was absent after 24 h. For SCCHN lines UM-SCC 11B and UM-SCC 22B, both examples chosen as good responders to 10 μM TAM, no decrease in PKC activity was seen at any determination interval (Table 4).

Discussion

The present study demonstrates significant antineoplastic activity of micromolar TAM concentrations in SCCHN cell lines, and as compared to the ER+ breast cancer line MCF-7, two head and neck carcinomas turned out to be even more sensitive to the anti estrogen. Growth inhibition analysis and apoptosis assays were indicative for a cytotoxic effect of high-dose TAM treatment in two SCCHN lines, whereas low-dose TAM caused apoptosis in MCF-7 cells only. Serum and tumor tissue concentrations of TAM around 1 μM are clinically achievable with standard dosages given in the adjuvant treatment of breast cancer.[27,28] Higher TAM concentrations between 5 and 10 μM, which in our as well as in other studies have been shown to induce growth inhibition in SCCHN cell lines,[33–35] can be obtained with high-dose regimens, such as performed in patients with malignant glioma[5,9,36] and other solid tumors.[29]

As yet, potential underlying mechanisms of the antineoplastic effect of TAM on SCCHN have been poorly investigated.[33–35] In established cell lines and surgical specimens of SCCHN, previous studies reported frequencies of ER expression between 0 and 69%.[37–45] This considerable range in receptor expression may be explained by the utilization of different determination methods and the lack of defined cut-off criteria.[39] The majority of studies employing biochemical as well as immunohistochemical methods found between 10 and 20% of SCCHN to be ER+.[37,41–45] In the present investigation, two well-established detection methods showed no evidence for ER expression in SCCHN cell lines, irrespective their obvious sensitivity to TAM. It is tempting, therefore, to speculate that other

Table 3. TGF-α, -β1 and -β2 concentrations (pg/ml) in tumor cell culture supernatants after 24 (values in brackets) and 72 h incubation with 0,1 or 10 μM TAM

Cell line	0 μM TAM			1 μM TAM			10 μM TAM		
	TGF-α (pg/ml)	TGF-β1 (pg/ml)	TGF-β2 (pg/ml)	TGF-α (pg/ml)	TGF-β1 (pg/ml)	TGF-β2 (pg/ml)	TGF-α (pg/ml)	TGF-β1 (pg/ml)	TGF-β2 (pg/ml)
UM-SCC 11B	10 (0)	1388 (1037)	624 (406)	40 (0)	1370 (1001)	587 (393)	20 (0)	1112 (1045)	360 (333)
UM-SCC 14C	0 (0)	855 (828)	114 (94)	0 (0)	880 (803)	111 (96)	0 (0)	839 (862)	110 (94)
UM-SCC 22B	0 (0)	926 (832)	195 (130)	0 (0)	903 (849)	200 (121)	0 (0)	956 (916)	141 (117)
8029 NA	20 (10)	965 (865)	1055 (348)	20 (20)	992 (833)	1135 (350)	20 (20)	892 (853)	660 (328)
8029 DDP	0 (0)	1210 (960)	930 (528)	0 (0)	1210 (930)	922 (530)	0 (0)	1090 (980)	783 (505)
MCF-7	60 (40)	770 (720)	132 (111)	40 (30)	840 (730)	137 (113)	30 (30)	830 (760)	137 (115)

Each growth factor was assayed in duplicate in three independent experiments. Results are specified as mean values and the SEM was generally $< 15\%$.

mechanisms than ER competition are responsible for TAM-mediated growth inhibition in these tumors.[4,7,21,46–48] Similarly, the mode of action underlying documented *in vitro* and *in vivo* antineoplastic activity of TAM in other ER– cancers is still under discussion.[3,5,8–12,21,29,36,49] Potentially relevant molecules which have been shown to be regulated and modulated by antiestrogens are peptide growth factors, including TGF-α, -β1 and -β2. However, in the SCCHN cell lines investigated, we observed no informative alterations of these growth factors in response to TAM exposure. On the other hand, TAM led to decreased TGF-α concentrations and slightly increased TGF-β1 levels in supernatants of ER+ MCF-7 cells. This is in accordance with other reports describing oppositional effects of TAM on growth-promoting TGF-α and antiproliferative TGF-β in ER+ cancer cell lines.[15,50] The significance of TAM-mediated estrogen antagonism for TGF-β1 upregulation has been nicely demonstrated in ER+ cancer cells which were either sensitive or resistant to estrogen.[16] It is remarkable, however, that TAM is also capable to stimulate TGF-β1 secretion in obviously ER– fibroblasts.[17,18] We finally looked for TAM-induced inhibition of PKC, a signal transduction enzyme manifold involved in the regulation of cell growth and differentiation, which has been particularly reported in ER– malignant glioma cells.[4,7,19,21] Neither TAM-responsive SCCHN cell lines nor MCF-7 cells showed conclusive alterations of PKC activity upon treatment with TAM. Since we determined total PKC activity, possible functional differences between cytosolic and membrane-bound enzyme were not considered.[20]

Conclusion

In summary, commonly suggested modes of action of TAM, such as competition with ER and interference with TGF-α, -β1, -β2 or PKC activity, do not appear to be responsible for the observed antitumor activity in cell lines of SCCHN. Thus, other mechanisms have to be taken into account, including alternative binding sites for TAM,[47,51,52] and less well-described interactions with intracellular signaling molecules like calmodulin, calcium and insulin-like growth factor.[53–56]

Despite apparent antineoplastic activity of TAM in SCCHN cell lines as demonstrated by us and others,[33–35] the application of TAM in the adjuvant treatment of SCCHN is far from being an established modality. In a first clinical study, no measurable

Table 4. PKC activity expressed as percentage compared to untreated control (100%) in the distinctly TAM-responsive cell lines MCF-7, UM-SCC 11B and UM-SCC 22B after 1, 24 and 72 h incubation with 10 μM TAM

	MCF-7	UM-SCC 11B	UM-SCC 22B
Untreated	100 $\pm$ 6.7	100 $\pm$ 19.2	100 $\pm$ 26.7
10 μM TAM/1 h	53.7 $\pm$ 5.6[a]	210.3 $\pm$ 55.9	ND
10 μM TAM/24 h	107.9 $\pm$ 12.1	137.2 $\pm$ 37.9	115.9 $\pm$ 26.5
10 μM TAM/72 h	ND	129.3 $\pm$ 19.5	83.8 $\pm$ 21.2

Results are specified as mean values of three independent experiments $\pm$ SEM. Values were tested in a paired *t*-test for significance ([a]$p \leqslant 0.05$).

responses were obtained in patients with pretreated SCCHN.[57] The protocol scheduled a well-tolerated standard dosage of 20 mg TAM/day which, however, is known to produce plasma levels below 1 μM.[27,28] According to our *in vitro* results this concentration should not be sufficient to induce significant antiproliferative and/or cytotoxic effects in ER– SCCHN. In order to achieve relevant tumor responses in HNSCC it may be necessary to substantially increase the dose of TAM in terms of a high-dose therapy as performed in other ER– solid type tumors.[5,29,36]

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