Tamoxifen inhibits the growth of head and neck cancer cells and sensitizes these cells to cisplatin induced-apoptosis: role of TGF-β1

Mahvash Tavassoli1,3, Jila Soltaninia1, Joanna Rudnicka1, Dorothy Mashanyare1, Newell Johnson1 and Joop Gäken2

1Head and Neck Oncology Group, Department of Oral Medicine and Pathology and 3Molecular Medicine, King’s, Guy’s and St Thomas’ School of Medicine and Dentistry, The Rayne Institute, 123 Coldharbour Lane, London SE5 9NU, UK
2To whom correspondence should be addressed at: Head and Neck Oncology Group, Guy’s, King’s and St Thomas’ School of Dentistry, The Rayne Institute, 123 Coldharbour Lane, London SE5 9NU, UK
Email: mahvash.tavassoli@kcl.ac.uk

A number of studies have shown that tamoxifen increases the sensitivity of several types of solid tumours to cisplatin without increasing the associated side effects. The cellular mechanisms responsible for this increased sensitivity are currently unknown. In this study we have investigated whether tamoxifen alone or in combination with cisplatin could induce apoptosis in head and neck squamous cell carcinoma (HNSCC) cell lines. We have shown that tamoxifen treatment resulted in G1 arrest in two cell lines, HN5 and HN6. Tamoxifen induced growth suppression was independent of p53 status but resulted in up-regulation of cyclin dependent kinase inhibitors (CDKIs) p21/Waf-1, p27/Kip1 and p15/INK4a. Furthermore, tamoxifen treatment resulted in an increased level of hypophosphorylated active RB. Cisplatin induced p53 independent apoptosis in both head and neck cancer cell lines. There was a significant sensitizing effect of tamoxifen on cisplatin-induced apoptosis in HN5 and HN6 cells, with the combined treatment being more effective in inducing apoptosis. Addition of tamoxifen did not result in significant inhibition of PKC activity in HN5 and HN6 cells. However, tamoxifen treatment resulted in increased secretion of TGF-β1 by HN5 and HN6 cells. An anti-TGF-β blocking antibody prevented both the blockade of cellular proliferation and the increased expression of CDKIs associated with tamoxifen treatment of HN5 and HN6 cells. These results show that tamoxifen alone induces a transient G1 arrest that greatly sensitizes the cells to apoptosis induced by cisplatin. We have shown that the mechanism for this p53-independent G1 arrest and apoptosis is at least partly due to the activation of TGF-β1 resulting in the induction of p15/INK4b, p27/Kip-1, p21/Waf-1 and RB hypophosphorylation. These in vitro results suggest that combination of tamoxifen and cisplatin might be a more effective treatment for head and neck cancers than single modality therapy.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) are amongst the most morbid of human cancers and affects nearly 500 000 patients annually world-wide (1). Standard treatment strategies involve surgery, radiotherapy and chemotherapy. Several chemotherapeutic agents have been used to treat HNSCC and combination chemotherapy regimens have been shown to induce positive responses in 30–40% of patients (2). However, treatment with current chemotherapeutic drugs does not substantially improve the survival rate. One reason for the poor response of head and neck cancers to chemotherapy is believed to be the high rate of p53 mutations in these tumours resulting in the loss of p53 induced growth arrest and/or apoptosis in response to DNA damage (3,4). To improve the poor survival rate there is a clear need to test combinations of drugs and to understand the mechanisms of their effects on the regulation of p53 independent growth inhibitory pathways in HNSCCs.

Cisplatin is an alkylating agent that binds to DNA and causes production of intrastrand cross-links and formation of DNA adducts. Cisplatin is an important agent which is used for the treatment of a wide range of cancers including breast, lung and head and neck (5). However, many patients rapidly acquire resistance to cisplatin during therapy. Proposed mediators of cisplatin resistance include the activation of the protein kinase C (PKC) signal transduction pathway and associated c-FOS overexpression (6). In a number of recent studies it has been shown that cisplatin in combination with other drugs is more effective than cisplatin alone and in combination with tamoxifen (TAM) it has been used for the treatment of several types of cancer (7,8).

TAM is a triphenylethylene anti-oestrogen first synthesized more than 20 years ago. It has been used extensively for the treatment of carcinoma of the breast. A high percentage of breast carcinomas express the oestrogen receptor (ER) which is blocked by tamoxifen. TAM also inhibits proliferation of ER negative breast carcinoma and glioma cells (9–11). The non-ER targets of TAM action include inhibition of PKC activity and enzyme calmodulin-dependent cAMP phosphodiesterase (CDP) which is crucial for cellular proliferation (12,13). Furthermore, the ER-independent growth inhibition of TAM has been shown to be via its effect on regulators of cell growth. TAM increases the secretion of negative growth regulators such as transforming growth factor β1 (TGF-β1) in human foetal stromal cell lines that lacked ER (14). The mechanism is believed to be via a post-translational action of TAM as there was no corresponding increase in TGF-β1 mRNA (14). Further in vivo studies with human breast carcancers confirmed increased amounts of TGF-β1 in the stroma of ER negative cancers after treatment with TAM (15).

TGF-β1 is a pleiotropic cytokine that can regulate the proliferation, development and functional activity of a wide range of cell types. Resistance to TGF-β1-mediated inhibition of cell proliferation is a common feature of many malignancies of lymphoid and epithelial origin and may contribute to their aberrant growth (16). TGF-β1-induced G1 arrest correlates with the transcriptional activation of cyclin dependent kinase
inhibitors (CDKIs) (17,18). CDKIs negatively regulate progression through the cell cycle by inhibiting phosphorylation and hence activation of cyclin-dependent kinases (cdks). There are two classes of CDKI; the inhibitor of cdk4 (INK4) family (p16/INK4a, p15/INK4b, p18 and p19) and kinase inhibitor protein (KIP) family (p21/WAF1/CIP-1, p27/Kip1 and p57Kip2) (19).

TGF-β1 regulates the activity of CDKIs in a cell type specific manner. In keratinocytes, p15/INK4b is upregulated 30-fold by TGF-β1 with no changes in p16/INK4a and p27/Kip1 expression (18). In gastric cancer cell lines, TGF-β1 up-regulates p21/Waf-1 ~3-fold, but the expression of p27/Kip1 is only slightly affected (20). A recent study demonstrated that TGF-β1 up-regulates p15/INK4b, p21/Waf-1 and p27/Kip1 and blocks cell cycling in G1 in human prostate epithelium (21). TGF-β1 induces G1 arrest through effects on the RB/E2F pathways. These results are consistent with the recent demonstration that TGF-β1 fails to inhibit the proliferation of RB-/- fibroblasts (22). Analysis of a number of malignant cells has correlated the insensitivity to TGF-β1 with the expression of mutant forms of p53 (23). This, and the demonstration that overexpression of mutant p53 can confer partial resistant to TGF-β1, suggests that p53 may also be involved in TGF-β1 growth inhibitory pathways. However, it has recently been shown that TGF-β1 induced growth control can exist independently of the presence of mutant p53 (20).

TAM increases the sensitivity of several types of solid tumours to cisplatin without increasing its side effects (24). TAM/cisplatin combination chemotherapy has been tested in phase II trials for melanoma, prostate, bladder, and non-small cell lung cancer (25-28). However, the mechanism for the sensitizing effect of TAM in these tumours is currently unknown. In this study we investigated the effect of TAM and cisplatin on the growth of two human tumour cell lines HN5 and HN6 both cellular targets which may mediate the effects of TAM in HNSCCs.

Materials and methods

Cell culture

HN6 and HN5 cells were grown in DMEM medium supplemented with 10% foetal calf serum. When required cells were treated with 4-hydroxyTAM (Promega) or cisplatin (50% dimethylformamide, 20% glacial acetic acid, 20 mM HCl and 20% ethanol). Cells were cultured in DMEM medium supplemented with 10% foetal calf serum. When required cells were treated with 4-hydroxyTAM or cisplatin.

Measurement of activated TGF-β1

Western blotting was performed as previously described (29). Samples were resolved by SDS–PAGE, 6% (RB) or 12% (p15, p21 and p27). The antibodies used were: p15/INK4b, R-20 (Santa Cruz); p21/Waf-1, EA10 (Oncogene Research products); p27/Kip1 (gift of Dr Xin Lu, Ludwig Institute for Cancer Research, St Mary’s Hospital Medical School, London) RB, G3-245 (Pharmingen); All the primary antibodies except the RB G3-245 were diluted 1:1000 in blocking buffer. The G3-245 antibody was diluted 1:500 in TBST. Secondary antibodies tagged to horse radish peroxidase were diluted 1:1000 in TBST. Secondary antibodies were detected using the enhanced chemiluminescence method (ECL) according to the manufacturer’s instructions (Amersham).

Cell cycle analysis by flow cytometry

Between 5×10³ and 10⁶ cells were harvested by trypsinization. The supernatant was also collected in order to analyse potential apoptotic bodies floating in the culture supernatant. Cells were collected by centrifugation at 200 g for 5 min. The cell pellet was washed in 1 ml PBS, transferred to microfuge tubes and collected by centrifugation at 200 g for 1 min. The cells were fixed by resuspension in 80% ethanol and incubated at -20°C for a minimum of 1 h and stored for a maximum of 1 month. For DNA staining the cells were harvested at 200 g for 1 min and were resuspended in 1 ml PBS containing 20 µg/ml RNase A and 20 µg/ml propidium iodide. Cell cycle distribution was analysed by flow cytometry using a FACSCAN analyser from Becton Dickinson.

Apoptosis was analysed by staining with Annexin V-fluores reagent (Boehringer Mannheim, 1828681) according to the manufacturer’s instructions. The cells and apoptotic bodies were harvested by trypsinization. The medium was removed, and cells were washed in 1 ml PBS. The cells were centrifuged at 200 g for 1 min and resuspended in 100 µl of Annexin V-flows labeling solution (10 mM Hepes pH 7.4, 140 mM NaCl, 5 mM CaCl₂, 20 µl Annexin V-flows, 20 µg/ml propidium iodide). The cells were incubated for 10–15 min after which the cells were diluted with 400 µl of incubation buffer (100 mM Hepes pH 7.4, 140 mM NaCl, 5 mM CaCl₂). Analysis was performed by flow cytometry.

PKC activity assay

PKC activity was measured using SigmaTTECT protein kinase C assay system (Promega V7470) using the conditions recommended by the manufacturers. Briefly 5 × 10⁶ cells were lysed in 400 µl of cold extraction buffer (25 mM Tris HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mM PMSF). The supernatant was passed over a 1 ml DEAE cellulose column pre-equilibrated in extraction buffer, and the column was washed with 5 ml extraction buffer. The PKC containing fraction was eluted with 5 ml extraction buffer containing 200 mM NaCl. Different dilutions of the eluted samples were incubated with PKC biotinylated protein peptide substrate buffer at 30°C for 5 min and then spotted on SAM biotin capture membrane. The membrane was washed in 2 M NaCl in 1% H₂PO₄. PKC activity was examined by scintillation counting.

Measurement of activated TGF-β1

The amount of TGF-β1 released into the cell culture supernatant of HNSCC cells was measured before and after treatment with 1 µM TAM using the Quantikine human TGF-β1 immunoassay system (R & D Systems). This ELISA assay measures TGF-β1 in cell culture supernatant. Briefly, TGF-β1 treated samples were incubated with TGF-β1 specific antibody (Monikine human TGF-β1 immunoassay system, R & D Systems) and monoclonal antibody and detected with horseradish peroxidase conjugated polyclonal antibody. The amount of TGF-β1 released into the cell culture supernatant was measured by chemiluminescence assay (ECL, Amersham).
induction of apoptosis in head and neck squamous cell carcinomas by combination therapy

soluble receptor Type II, which binds TGF-β1 has been pre-coated onto a microtitre plate. Standards and samples are added to the wells and any TGF-β1 present is bound by the immobilized receptor. After washing any unbound substrates, an enzyme-linked polyclonal antibody specific for TGF-β1 is added to the wells to sandwich the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells, colour develops in proportion to the amount of TGF-β1 bound in the initial step. The colour development is stopped and the intensity of the colour is measured in pg/ml.

Cells were grown to subconfluence in 12-well dishes containing 1 ml of complete medium. Standards and samples in triplicate were taken after 0, 24, 48 or 72 h of exposure to TAM and added to the precoated microplates. The assay was performed according to the manufacturer’s recommendation using standards provided with the kit.

results

Effect of TAM and cisplatin on proliferation and cell cycle distribution of head and neck cancer cells

Head and neck cancer cell lines, HN5 and HN6 were treated with several different concentrations of TAM. Growth rate measured by MTT assay showed that 1 µM TAM reduced the proliferation rate of HN5 and HN6 cells compared with the same cells cultured in an identical concentration of ethanol, the solvent used to dissolve TAM (Figure 1).

We also studied the effect of cisplatin on the proliferation of these same cell lines. Treatment with 10 µg/ml cisplatin resulted in almost 50% inhibition of proliferation of HN5 and HN6 cells (Figure 1). Interestingly, combined treatment with 1 µM TAM and 5 µg/ml cisplatin significantly enhanced the growth inhibitory effects on HN5 and HN6 cells compared with treatment with each drug individually.

To analyse the effect of TAM and/or cisplatin on cell cycle distribution, HN5 and HN6 cell lines were treated for 72 h with either TAM or cisplatin alone or a combination of TAM/cisplatin. Cisplatin at a concentration of 10 µg/ml showed a significant toxicity, we therefore used 5 µg/ml cisplatin in the experiments where we used combinations with TAM.

Cellular DNA was stained with propidium iodide and cell cycle distribution was studied by FACS analysis. Both HN5 and HN6 cells treated with 1 µM TAM, showed more cells arrested in G1 (Figure 2). Treatment of these cells for 72 h with 5 µg/ml cisplatin alone resulted in increased apoptosis detected by the appearance of a sub-G1 peak (Figure 2). When cells were treated with both cisplatin and TAM for 72 h apoptosis increased in HN5 and HN6 cells (see Figure 2 and Table I). This result shows that even though TAM alone induces a transient G1 arrest, it greatly sensitizes the cells to apoptosis induced by the DNA damaging drug cisplatin.

To investigate if the observed sub-G1 peak was due to apoptosis, cells were stained with Annexin V conjugated to FITC which only binds apoptotic cells. The results of flow cytometry summarized in Table I showed that combined cisplatin/TAM treatment for 48 h resulted in higher levels of apoptosis compared with single treatment with cisplatin.

Effect of TAM and cisplatin on p21/Waf-1, p15/INK4b, p27/Kip1 and RB

We have shown that the two HNSCC cell lines used in this study have mutations in the p53 gene and also mutation in p16/INK4a (29). To study if cell cycle arrest and apoptosis, induced by these drugs, affected the expression of cell

![Fig. 2. Cell cycle analysis of HN5 and HN6 cells after treatment with TAM and cisplatin. Cells were treated with (either 1 µM TAM, 5 µg/ml cisplatin or combination of both) for 48 h. Cells were fixed and stained with propidium iodide (PI) before flow cytometry analysis. Fractions of cells at apoptosis (Ap) and G1 are indicated on each profile.](https://academic.oup.com/carcin/article-abstract/23/10/1569/2896619/1571)
Table I. Effect of TAM and cisplatin on HN5 and HN6 cells analysed by annexin V staining and FACS

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<th>HN5 (%) Apoptotic</th>
<th>HN6 (%) Live</th>
<th>HN6 (%) Apoptotic</th>
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Cells were stained with annexin V and propidium iodide and cell cycle distribution was analysed by flow cytometry. Each value is the average of three independent experiments. Columns show percentages of cells at each stage of the cell cycle: T, tamoxifen; C, cisplatin.

Fig. 3. Induction of protein expression by TAM and cisplatin. Western blot analysis was performed on cell extracts prepared from HN5 and HN6. Cells were treated with 1 µM TAM, control cells (C) were treated with similar concentration of ETOH for 48 h. 50 µg total cell lysate was analysed for the expression of p15/INK4b, p21/Waf-1, p27/Kip 1 or RB.

cycle checkpoint genes we measured the levels of p15/INK4b, p21/Waf-1, p27/Kip 1 and RB. The cells were treated with 1 µM TAM or 5 µg/ml cisplatin for 72 h and western blot analysis was performed on total cell extracts (Figure 3). In HN5 cells the levels of p15/INK4b and p21/Waf-1 protein but not p27/Kip1 increased with 1 µM TAM (Figure 3). Treatment of HN6 cells with 1 µM TAM induced increased levels of p21/Waf1 and p27/Kip1 but not p15/INK4b. Control HN5 and HN6 cells express both hypo- and hyperphosphorylated RB. Addition of TAM resulted in increased levels of hyperphosphorylated active RB in relation to hyperphosphorylated inactive RB in both cell lines. The levels of p15/INK4b and p27/Kip1 was maximal after 48 h while p21/Waf-1 and active hyperphosphorylated RB levels remained elevated at 72 h. Cisplatin treatment of HNSCC cells at 5 µg/ml failed to induce p15/INK4b or p21/Waf-1 and p27/Kip 1 after 3 days (data not shown).

Effect of TAM on PKC and TGF-β1 activity in HN5 and HN6 cells

To investigate whether growth inhibition associated with TAM treatment of HN5 and HN6 cells was due to the inhibition of PKC activity, cells were treated with either 1 µM TAM or 100 nM PKC inhibitor, staurosporine (Calbiochem). PKC activity was measured after 72 h as described in Materials and methods. The results showed no significant change in the level of PKC activity in HN5 and HN6 cells treated with TAM (Figure 4) whilst cells treated with 100 nM staurosporine showed complete inhibition of PKC activity.

TAM has been shown to induce expression of TGF-β1 in cells lacking oestrogen receptors. We explored the possibility that TAM induction of CDKIs and hypophosphorylation of RB may be due to increased activity of TGF-β1.

First we tested whether HNSCC cells were responsive to the negative growth effects induced by TGF-β1. Recombinant TGF-β1 protein was added to the culture medium at concentrations of 0, 1, 10, 30 and 100 pM. Tritiated thymidine incorporation assays showed inhibition of cell proliferation in both HN5 and HN6 cells after addition of TGF-β1. The maximum growth arrest was observed after 72 h treatment with 100 pM TGF-β1 (Figure 5A). Western blot analysis showed increased levels of p21/Waf-1 and p15/INK4b in HN5 and p21/Waf-1 and p27/Kip 1 in HN6 cells treated with TGF-β1 (Figure 5B).

To investigate the secretion of TGF-β1 by HNSCC cells triplicate samples were treated with 1 µM TAM and levels of the secreted form of TGF-β1 were measured after 24, 48 and 72 h as described in the Materials and methods section. The level of TGF-β1 increased more than two fold in HN5 and HN6 cells after 24 h treatment with TAM (Figure 6). This increase reached 3-fold 72 h after treatment in HN5 and HN6 cells.

TGF-β blocking antibody prevents TAM-induced growth inhibition

The increased TGF-β1 level induced by TAM led us to investigate whether TGF-β receptor activity was required for the growth inhibition of HN5 and HN6 by TAM. We measured uptake of [3H]-thymidine in cells treated with TAM or solvent alone (ETOH) in the absence or presence of anti-TGF-β blocking antibody. As shown in Figure 7A, uptake of [3H]-thymidine was inhibited by about 30% in HN5 and HN6 cells treated with 1 µM TAM compared with cells treated with solvent alone (ETOH). Anti- TGF-β blocking antibody alone did not inhibit [3H]-thymidine uptake. However, anti-TGF-β1 blocking antibody significantly reversed the inhibitory effect of TAM on both HN5 and HN6 cells. These data demonstrate...
that TGF-β is involved in inhibition of cellular proliferation by TAM in HN5 and HN6 cells.

Since we had shown that both TAM and TGF-β treatment resulted in up-regulation of CDKIs we investigated whether anti-TGF-β blocking antibody affected this up-regulation. Western blotting was performed on cell extracts prepared from HN5 and HN6 cells after treatment with TAM in the presence or absence of TGF-β blocking antibody. Forty-eight hours after treatment, cells were harvested and 50 µg total cell lysate was analysed for the expression of p15/INK4b, p21/Waf1 and p27/Kip1. As shown in Figure 7B, treatment with TGF-β blocking antibody prevented the induction of p15/INK4b and p27/Kip1 and significantly reduced induction of the p21/Waf1 level by TAM.

**Discussion**

In this study we have analysed the effect of TAM alone or in combination with cisplatin on HNSCC cells. Furthermore, we have investigated the pathways which may be involved in the response of HNSCC cells to TAM and/or cisplatin. TAM is a widely used drug for adjuvant therapy in the treatment of breast cancer and has a low incidence of serious side-effects. Recent preclinical and clinical findings show an increased effect of TAM in combination with different cytotoxic agents in the treatment of melanoma and other cancers (9). To extend these studies we investigated the effect of TAM on head and neck cancer cell lines. The effect of TAM on head and neck cancers has not been previously studied.

In this study we have shown that TAM induced a transient G1 growth arrest in HN5 and HN6 head and neck cancer cells. Both lines have mutations in the p53 gene and therefore lack p53 dependent growth arrest. The gene encoding p16/INK4a is also mutated in these cell lines suggesting that regulation of RB activity may be impaired. Treatment of HN5 and HN6 cells with TAM induced expression of CDKIs p21/Waf1, p15/INK4a and p27/Kip1 and resulted in an increased level of hypophosphorylated, active RB. Cisplatin alone induced apoptosis in both HN5 and HN6 cells. Cisplatin treatment had no significant effect on the level of CDKIs. It is therefore unclear which pathways are involved in apoptosis induced by cisplatin.

When HN5 and HN6 cells were treated with a combination of TAM and cisplatin, the combined drug treatment resulted in a much stronger induction of apoptosis than could be achieved with either drug alone. This increased apoptosis was clearly observed after 48 h of combined treatment and coincided with the increased levels of CDKIs. The combined effect of these two drugs therefore seems to be due to the increased
levels of CDKIs induced by TAM whilst cisplatin probably activates other pathways involved in the induction of apoptosis.

The inhibition of tumour cell growth by TAM in breast cancers is believed to be due to inactivation of oestrogen receptors (ER). However, TAM has also been shown to inhibit the growth of other tumours including lung, prostate bladder and ER negative breast cancer (10,11). The inhibition of protein kinase C, angiogenesis inhibition and TGF-β stimulation are suggested as alternative ways through which TAM suppresses tumour cell growth independently of the expression of ER (13,14,30). We therefore investigated if the G1 growth arrest induced by TAM in the head and neck cancer cell lines HN5 and HN6 cells was associated with inhibition of PKC activity and or increased activation of TGF-β1.

Treatment of HN5 and HN6 cells with TAM had no significant effect on the total PKC activity. To explore the role of TGF-β1 in the growth inhibition by TAM, we tested if HN5 cells were responsive to the growth inhibitory effect of TGF-β1. Treatment of HN5 and HN6 cells with 100 pM TGF-β1 for 72 h resulted in more than 50% growth inhibition of these cells. Western blot analysis showed increased levels of CDKIs and increased levels of hypophosphorylated pRB in both HN5 and HN6 cells in response to TGF-β1.

To examine if TAM treatment resulted in production of natural TGF-β1 protein before and after treatment of HN5 and HN6 cells with TAM. The level of TGF-β1 increased 2-fold after 24h treatment with TAM. This increased level was sustained for up to 72 h, the duration of the experiment. This early induction suggest that an increase in the TGF-β1 level may precede and be responsible, at least in part, for the observed increase in the levels of p15/INK4b, p21/Waf1 and hypophosphorylated RB in HN5 cells. To investigate whether increased levels of TGF-β1 were responsible for the anti-proliferative effects of TAM we used an anti-TGF-β1 blocking antibody. The demonstration that TAM induced growth inhibition of HN5 and HN6 cells as well as induction of CDKIs were prevented by this blocking antibody further suggest an important role for TGF-β1 in the growth inhibition by TAM. However, this antibody did not completely reverse TAM induced inhibition of cellular proliferation suggesting that TGF-β1 independent pathways may also be involved in the inhibition of cellular proliferation observed with TAM treatment.

In conclusion our findings demonstrate that, in vitro, TAM has a growth inhibitory effect on head and neck carcinoma cells which is independent of p53 and p16/INK4a functions. This growth inhibition seems to be independent of PKC activity in contrast with other cancer cell lines such as malignant gliomas where TAM downregulates PKC. The growth inhibitory effect of TAM on HNSCC cells seems to be mediated via up-regulation of TGF-β1. The results of this in vitro study helps to understand the pathways which may be involved in the uncontrolled proliferation of HNSCC cells and the information obtained may facilitate the design of more effective drug or gene therapy strategies for the treatment of head and neck cancers.
Acknowledgements

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References


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