

Ectopic Expression of Protein Kinase C- β Sensitizes Head and Neck Squamous Cell Carcinoma to Diterpene Esters

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Abstract. *Background:* The objective of this study was to examine the effect of specific Protein kinase C (PKC) isoform re-expression in solid malignancies, particularly head and neck squamous cell carcinoma cell lines, and the impact this may have on treatment with known activators of PKC. *Materials and Methods:* The constitutive expression of PKC isoforms were determined in six head and neck squamous cell carcinoma (SCC) cell lines. Cytotoxicity of the prototypic phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) and the novel diterpene ester PEP005 was established. Viral transduction to re-express PKC β isoforms in two of these cell lines was performed, and its effect on the sensitivity to the compounds was quantified. *Results:* Tongue and hypopharyngeal SCC cell lines were resistant to both TPA and PEP005, with the concentration required to inhibit growth by 50% (IC₅₀) being >1,000 ng/ml. CAL-27 (tongue SCC) and FaDu (hypopharyngeal SCC) cell lines re-expressing PKC β I and - β II isoforms demonstrated IC₅₀ of 1-5 ng/ml with TPA or PEP005. *Conclusion:* Re-expression of PKC β in head and neck SCC cell lines leads to cells one thousand-times more sensitive to the cytotoxic effects of phorbol or diterpene esters in culture. This highlights the importance of the isoform in tumor progression and presents the potential benefit of these compounds in malignancies expressing the protein, and in combination therapy.

Head and neck cancer is the fifth most common cancer, with an estimated global incidence of more than 500,000 cases

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Key Words: Protein kinase C, HNSCC, TPA, PEP005, diterpene.

annually (1). Squamous cell carcinoma comprises approximately 90% of all head and neck cancer (2) and despite advances in surgical techniques, drug discovery and radiotherapy, the five-year survival rates for patients with head and neck squamous cell cancer (HNSCC) have improved only marginally in the past 30 years (3), remaining at around 50%, with a median survival of six months for patients with local recurrence or metastatic disease (4). Currently, the most effective treatment option for non-oropharyngeal, human papillomavirus-positive solid tumors is surgical resection followed by adjuvant radiotherapy with/without chemotherapy to minimize the risk of recurrence (5). However, failure of locoregional control of HNSCC is common, with approximately 60-70% of patients experiencing local tumor recurrence following conventional surgery and radiotherapy (6). In addition, patients with tumor recurrence are often not candidates for re-resection or re-irradiation due to excessive morbidity associated with surgical excision in previously irradiated fields.

Protein kinase C (PKC) is a family of serine/threonine kinases which are involved in an array of cell signalling pathways which dictate cellular processes such as proliferation, differentiation, migration and apoptosis. Since their discovery in the early 1980s, twelve isoforms of PKC have been identified; characterized by their structure and activating substrates, they have been subdivided into three groups. The conventional PKCs, - α , - β I, - β II and - γ are calcium-dependent and activated by diacylglycerol (DAG), phosphatidylserine (PS) and phorbol esters. The novel PKCs - δ , - ϵ , - η and - θ are also activated by DAG, PS and phorbol esters but are however independent of calcium. Finally, the atypical PKCs, - ζ and - ι , are activated by cis-unsaturated fatty acids and are also calcium-independent. All the PKC isoforms do however share a common basic structure, a single polypeptide with both a cell membrane-targeting N-terminal regulatory region, and a C-terminal catalytic region. Following the

loss of pseudo-substrates from the catalytic C-terminus, the enzyme alters its shape and adopts an open configuration and is then recruited to the plasma membrane for activation by its respective substrate.

PKC isoforms are known to play either an oncogenic or a tumor-suppressing role in cell-cycle regulation, however these effects have been found to be rather cell-specific, thus tumor cell PKC isoform profiling has become important in order to determine which isoforms are down-regulated or overexpressed in certain tumor types. The outcome of PKC isoform activation is intimately linked to activation of signalling pathways within the cell, including the activation of the mitogen-activated protein kinase (MAPK) pathway. MAPK is a known key regulator of cell proliferation, differentiation, and survival, as well as of apoptosis, and can play a central role in growth inhibition. Recently, novel PKC activators have been shown to selectively induce p21 expression in sensitive cells, *via* the activation of the MAPK pathway, causing a G₁ arrest by inhibiting multiple cyclin-dependent kinases thus leading to the hypo-phosphorylation of the retinoblastoma protein, which, in turn, inhibits S-phase progression (7-9). However, their full potential has not yet been widely exploited (9). These novel compounds, diterpene esters, are thought to exert their function by imitating the binding of the endogenous ligand DAG, and allowing PKC to dock onto membranes and access its substrates (10). Diterpene esters have also been shown to have anti-neoplastic properties (11). The discovery of diterpene esters and their impact on tumor cell death, senescence and innate immune system stimulation *via* PKC activation (12) has provoked much investigation into their role in solid tumor regression and eradication.

The conventional isoform PKC β , is well-known to be underexpressed or down-regulated in many tumour types including of the bladder and colon, melanoma and HNSCC. The vast majority of the work done in recent years with PKC β , and its role in tumorigenesis, has exposed its importance in endothelial cell function and its action as a promoter of angiogenesis and tumour growth. Conversely Oka *et al.* have also highlighted its participation in melanoma as a tumor suppressor, resulting in reduced invasion and increased apoptosis (13). This contrast raises the probability that PKC β has the propensity to act as either a promoter or a suppressor of malignant cellular pathways.

The aim of the current study was to assess the constitutive expression of PKC isoforms in a panel of HNSCC cell lines, and to establish cytotoxicity of the prototypic phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) and the novel diterpene ester PEP005 (ingenol mebutate) in these lines. We then aimed to assess the effect of re-expression of PKC isoforms (PKC β I and - β II) on sensitivity to the compounds in HNSCC cells.

Materials and Methods

Cell lines, cell culture and materials. All cell lines used in this study were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) (tongue SCC: CAL-27, SCC-9, SCC-15, and SCC-25; hypopharyngeal SCC: FaDu; nasal septum SCC: RPMI-2650; epidermoid carcinoma: A-431; vulval SCC: SW-954 and SW-962). Cells were cultured in 5% CO₂ at 37°C (Stericult 200; Forma Scientific, Marietta, OH, USA) in RPMI-1640 (CSL Biosciences, Parkville, Victoria, Australia) containing 10% v/v foetal calf serum (FCS; CSL Biosciences), 3 mM 4-(2-hydroxyethyl)-piperazine ethane sulphonic acid (HEPES), 60 µg/ml penicillin (CSL Biosciences) and 100 µg/ml streptomycin (CSL Biosciences). All cell lines were passaged at 90% confluence. Detachment of adherent cells was performed using a solution containing 0.25% (v/v) trypsin (Invitrogen; Carlsbad, CA, USA), 3.8% (v/v) versene in Dulbecco's phosphate buffered saline (PBS) solution following a wash in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄; pH 7.4). 12-O-tetradecanoylphorbol-13-acetate (TPA), PEP005 (ingenol mebutate) and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell survival assays. Cultured cells were counted using a haemocytometer and seeded at 3×10⁴ cells per microliter in RPMI-1640 with 10% FCS in flat-bottom 96-well plates. Plates were incubated for 24 h and then treated with compounds, as described below. Cells were allowed to grow until control wells were nearly-confluent. SRB was used as a basic protein stain to compare the growth of cells treated with specific agent, with the growth of untreated or differently treated cells as a measure of cell number. Media were discarded, wells were washed once with PBS to remove excess FCS, and cells were then fixed with methylated spirits before being washed with tap water and stained with 50 µl/well of 0.4% SRB in 1% acetic acid. After 15 minutes, the SRB was discarded and the plates were washed with 1% acetic acid three times. Finally, 100 µl of 10 mM Tris base (unbuffered) was added to each well before reading the absorbance at 564 nm on an ELISA reader (VERSA max tuneable microplate reader; Molecular Devices, Sunnyvale, CA, USA) with a 3 s prior shaking. An average blank absorbance of 0.04 AU was subtracted from all experimental data.

Total cell lysates and western blot analysis. Total cell lysates from 1×10⁷ cells were generated as previously described (14). Thirty micrograms of sample protein loaded in sodium dodecyl sulphate (SDS)-loading buffer [100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol, 100 mM dithiothreitol (ICN, Aurora, OH, USA), 10% phenylmethylsulfonyl fluoride (Sigma-Aldrich)] was resolved by 10% SDS-polyacrylamide gel electrophoresis at 200 V for 45 min at room temperature, and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked with Blotto [5% w/v non-fat dry milk (Diploma, Melbourne, Australia) 0.5% Tween 20 in PBS], for 90 min at RT. Blots were then incubated with the primary antibody against PKC (antibody sampler kit cat. no. 611421; BD Biosciences, San Jose, CA, USA) and to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; R & D Systems, Minneapolis, MN, USA) as directed by the manufacturers in 2 ml of Blotto overnight at 4°C on a rotator. Blots were then washed with 0.5% v/v Tween-20 in

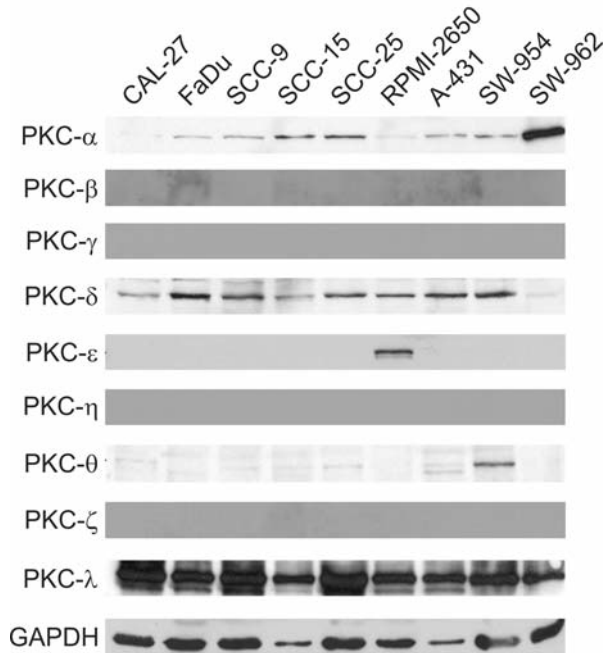


Figure 1. Constitutive expression of Protein kinase C isoforms in head and neck squamous cell carcinoma cell lines. Cell lysates (30 μ g) were analyzed by western blot. Images are representative of duplicate experiments.

PBS (2 \times 2 min; 3 \times 5 min) on an orbital shaker (Bioline, Alexandria, NSW, Australia) and incubated with the appropriate secondary antibody made up in 2 ml Blotto for 90 min at RT on the rotator. Blots were then washed as previously with 0.5% Tween 20 in PBS and antibody detection was performed using the enhanced chemiluminescence detection method (Western Lightning ECL Pro; PerkinElmer, Waltham, MA, USA). Following a one-minute incubation in 750 μ l of Enhanced Luminol Reagent and 750 μ l of Oxidising Reagent, blots were imaged using X-OMAT autoradiography film (Kodak; Rochester, NY, USA).

Generation of stable PKC β -expressing cell lines. Stable expression of PKC β I or - β II was achieved using the ViraPower™ Lentiviral Expression System (Invitrogen, Carlsbad, CA, USA). Briefly, PKC β I or - β II were cloned into pLenti6/V5-DEST using Gateway recombination. Lentivirus particles containing pLenti6/PKC β I or - β II were packaged in 293FT cells (Invitrogen), before being titred using MM96L cells. The negative control construct was identical except that it encoded the β -galactosidase (lacZ) gene (Invitrogen). Target HNSCC cell line pools were transduced with particles using a multiplicity of infection of less than 1 and selected for three weeks with 3 μ g/ml blasticidin (Invitrogen). All cells were maintained on 0.5 μ g/ml blasticidin for all experiments.

Determination of sensitivity to phorbol or diterpene esters. HNSCC cells, either untransduced lines or transduced to re-express PKC β I or PKC β II, or lacZ as a negative control, were plated as above, and then treated with either TPA or PEP005 at concentrations from 1 μ g/ml to 10 μ g/ml. Controls were treated with the equivalent volume of the solvent used to carry the

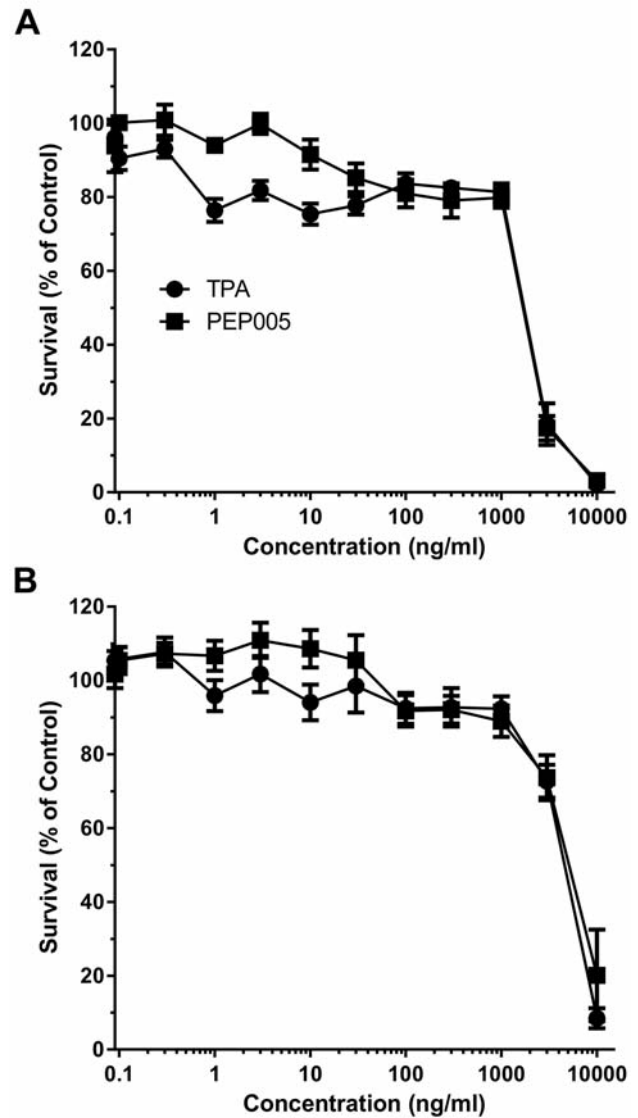


Figure 2. Head and neck squamous cell carcinoma cell line survival after treatment with phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or diterpene ester PEP005. HNSCC cells (3,000) were plated into 96-well flat-bottom plates and allowed to adhere for 24 h. CAL-27 (A) and FaDu (B) cells were then treated with the indicated amounts of TPA (circles) or PEP005 (squares) and incubated for five days before cellular cytotoxicity was assessed using sulfarhodamine B staining. Controls were treated with the same concentration of vehicle. Results are mean \pm standard deviation from triplicate readings from three independent experiments.

compounds (100% ethanol). Cells were allowed to grow until control treatments were 95% confluent (approximately 6 days), before cell survival assays were performed as above to determine survival following exposure to phorbol or diterpene esters. All results shown are mean \pm standard deviation from triplicate readings from three independent experiments.

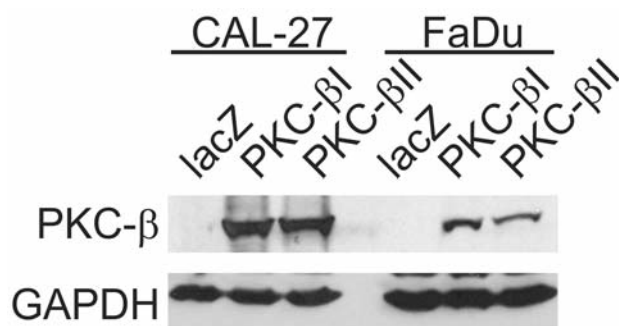


Figure 3. Ectopic expression of Protein kinase C β I or - β II in head and neck squamous cell carcinoma cell lines. CAL-27 and FaDu cells were transduced with lentiviral particles for expression of PKC β I, PKC β II or lacZ as a negative control.

Results

PKC isoform profiling in HNSCC. We profiled the expression of PKC isoforms in six HNSCC cell lines, as well as three other SCC cell lines of cutaneous origin by western blot analysis (Figure 1). Five of the HNSCC cell lines, four of which originate from the tongue (CAL-27, SCC-9, -15 and -25) and one from the hypopharynx (FaDu), share similar expression patterns of PKC isoforms. These cells all express a single conventional PKC enzyme in the form of PKC α and a novel isoform, PKC δ . There was detectable expression of PKC θ among the HNSCC cells, however, this level of expression was not considered significant enough to warrant further investigation in this study. In RPMI-2650 cell line, also an HNSCC variant but derived from a nasal septal primary carcinoma, although similarly significantly expressing PKC δ , expression of PKC ϵ was also detected. The three additional cutaneous SCC cell lines, A-431, an epidermoid carcinoma, and SW-954 and SW-962, both derived from vulval SCC, showed varying isoform expression, with the epidermoid carcinoma having an almost identical profile to the HNSCC lines, SW-954 expressing PKC α , - δ and - θ , and SW-962 only expressing the PKC α isoform. The atypical isoform PKC ι was uniformly expressed across all cell lines. In agreement with previous studies, all of the cancer cell lines lacked detectable expression of PKC β , and of isoforms - γ , - η , and - ζ .

In vitro cell survival of CAL-27 and FaDu cell lines following treatment with TPA. To determine the *in vitro* cytotoxicity of phorbol and diterpene esters in HNSCC, all cells were treated with TPA and PEP005 at doses ranging from 0.1 ng/ml to 10,000 ng/ml in clonogenic cell survival assays. The results show that cells treated with either TPA or PEP005 exhibited no apparent cytotoxicity at doses lower than 1,000 ng/ml, after which, cell survival numbers declined rapidly (Figure 2 and

data not shown). At a dose of 3,000 ng/ml, CAL-27 cell survival dropped to 18.4% (\pm 17.09%; standard deviation) when exposed to TPA, reducing to 2.1% (\pm 1.4) at 10,000 ng/ml. At 3,000 ng/ml, 17.4% (\pm 8.2) of cells survived when exposed to PEP005, while 3.1% (\pm 1.4) survived at 10,000 ng/ml. FaDu cells followed a similar trend, with 73.6% (\pm 15.5) survival at 3,000 ng/ml of TPA, dropping to 8.4% (\pm 9.5) with 10,000 ng/ml, and 73.7% (\pm 17.3) survival at 3,000 ng/ml and 20.1% (\pm 35.0) at 10,000 ng/ml PEP005. Importantly, the concentration required to inhibit growth by 50% (IC₅₀) were >1,000 ng/ml in each cell line for both TPA and PEP005.

Ectopic expression of PKC β isoforms in HNSCC cell lines. We wished to study the effect of overexpression of a PKC isoform in relation to sensitivity to cytotoxicity induced by PKC-activating compounds. We, therefore, constructed two HNSCC cell lines overexpressing either PKC β I or PKC β II where there was no detectable expression of these isoforms constitutively. CAL-27 and FaDu cells were transduced by lentiviral particles to enable expression of PKC β I or PKC β II, or lacZ as a negative control.

Western blot analysis of PKC β -transduced variants of the CAL-27 and FaDu cell lines was performed, probing for PKC β expression (Figure 3). A significant expression of the isoforms was detected in both cell lines transfected with each subtype of PKC β , confirming successful overexpression of the isoform in the two cell lines. Cells transduced with lentiviral particles containing a lacZ construct showed no increased expression of PKC β isoforms. Assays of cell growth of the PKC β I and PKC β II overexpressing CAL-27 and FaDu cell lines showed no significant difference when compared to those cells expressing lacZ (data not shown).

Ectopic expression of PKC β I or PKC β II sensitizes to killing by PKC-activating compounds. The sensitivity of HNSCC ectopically-expressing either PKC- β I or PKC- β II isoforms to PKC activating compounds was assessed. CAL-27 cell lines overexpressing PKC β isoforms depict a similar dose-response curve to each other (Figure 4A and B). The IC₅₀ for TPA and PEP005, respectively, for CAL-27 cells ectopically expressing PKC β I was 0.801 \pm 0.079 (standard error) and 1.40 \pm 0.143 ng/ml (Figure 4A and B). Ectopic expression of the PKC β II isoform in CAL-27 cells led to IC₅₀ of 0.705 \pm 0.064 and 1.29 \pm 0.086 ng/ml for TPA and PEP005, respectively (Figure 4A and B). For CAL-27 cells expressing lacZ, an IC₅₀ concentration was not reached for either TPA or PEP005.

The FaDu PKC β I- and PKC β II-transduced cell lines (Figure 4C and D) showed differences in sensitivity to TPA and PEP005. For FaDu cells ectopically expressing PKC β I, the IC₅₀ concentration was 1.28 \pm 0.156 (standard error) and 2.81 \pm 0.203 ng/ml for TPA and PEP005, respectively (Figure 4C and D), while for cells expressing PKC β II, the

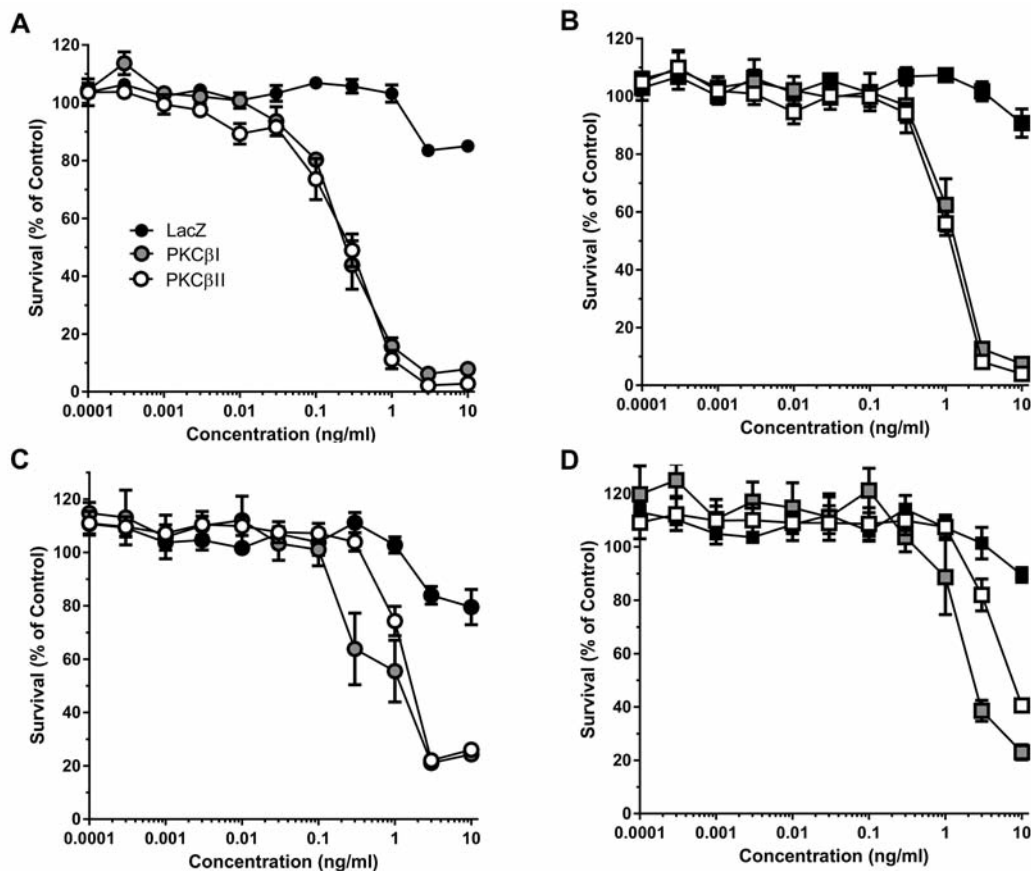


Figure 4. Ectopic expression of Protein kinase C β I or - β II in head and neck squamous cell carcinoma cell lines sensitizes them to phorbol ester-induced cytotoxicity. Cell survival of transduced CAL-27 (A, B) and FaDu (C, D) cell lines in cultures treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (A and C) or PEP005 (B and D). Controls were treated with the same concentration of vehicle. Results are mean \pm standard deviation from triplicate readings from three independent experiments.

corresponding values 2.36 ± 0.181 and 3.67 ± 0.259 ng/ml (Figure 4C and D). As observed for CAL-27 cells, an IC₅₀ for treatment with TPA and with PEP005 was not reached with FaDu cells expressing lacZ (Figure 4C and D).

Discussion

In recent years, there has been increasing interest in the potential of PKC activators as agents or adjuvants in cancer chemotherapy (15-17). TPA has previously been trialed for haematological malignancies, with modest responses (18, 19). PEP005 (also known as ingenol mebutate) has currently completed phase III clinical trials for the topical treatment of actinic keratosis (20) and phase II trials for non-melanoma skin cancer (21).

It has been well-established that PKC β is absent in multiple tumor types, including HNSCC, which was confirmed in the cell lines used in the present study. Ectopic expression through viral transduction of PKC β I or

PKC β II in CAL-27 and FaDu cell lines as representatives of HNSCC did not lead to visible differences in cellular morphology nor to differences in cellular growth rates. However, this study demonstrated that PKC β isoforms have the potential to function as tumor-suppressing variants when activated in HNSCC. Our results demonstrate that when the PKC β isoforms were ectopically expressed in HNSCC cell lines, the resulting cell lines became more sensitive to the prototypic phorbol ester TPA and the novel diterpene ester PEP005. PKC β -expressing HNSCC cell lines required approximately one thousandth of the dose required to induce cell death in culture following treatment with these compounds. These findings open an avenue for future investigations and identify PKC β as a possible therapeutic target in HNSCC. Although common, the complete absence of PKC β in cancer is not universal. Thus patients who suffer from malignancies that are known to express PKC β may be rapidly and effectively treated with known PKC activators such as phorbol or diterpene esters. Additionally, compounds

that lead to re-expression of methylated PKC β [e.g. 5-aza-2'-deoxycytidine, (22, 23)] could be used in combination treatment of HNSCC.

Acknowledgements

Parts of this work were supported by the National Health and Medical Research Council of Australia (NHMRC) Development Grant APP1017676 (PGP and GMB), and the Queensland Head and Neck Cancer Centre.

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Received November 27, 2014

Revised December 10, 2014

Accepted December 12, 2014