Original Research Article

Dacomitinib and gedatolisib in combination with fractionated radiation in head and neck cancer

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Background and purpose: There has been little success targeting individual genes in combination with radiation in head and neck cancer. In this study we investigated whether targeting two key pathways simultaneously might be more effective.

Materials and methods: We studied the effect of combining dacomitinib (pan-HER, irreversible inhibitor) and gedatolisib (dual PI3K/MTOR inhibitor) with radiation in well characterized, low passage xenograft models of HNSCC in vitro and in vivo.

Results: Dacomitinib showed differential growth inhibition in vitro that correlated to EGFR expression whilst gedatolisib was effective in both cell lines. Neither agent radiosensitized the cell lines in vitro. In vivo studies demonstrated that dacomitinib was an effective agent alone and in combination with radiation whilst the addition of gedatolisib did not enhance the effect of these two modalities despite inhibiting phosphorylation of key genes in the PI3K/MTOR pathway.

Conclusions: Our results showed that combining two drugs with radiation provided no added benefit compared to the single most active drug. Dacomitinib deserves more investigation as a radiation sensitizing agent in HNSCC.

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1. Background and significance

The EGFR/PI3K/AKT/mTOR signaling pathway plays a central role in numerous important cellular processes, including growth, proliferation, differentiation, migration, inflammation and survival under normal physiological and pathophysiological conditions such as cancer [1]. Genomic characterization of head and neck squamous cell cancer (HNSCC) has identified mutations in many genes that converge on the EGFR/RAS/RAF/ERK/PI3K/AKT/mTOR signaling cascade [2,3] and molecular alterations in one or more components of this pathway are present in more than 80% of HNSCC [4,5]. As a result, this signaling pathway is considered to be a very attractive target for molecular-oriented therapy [6]. However, apart from the inhibitors of EGFR and vascular endothelial growth factor (VEGF), very few molecular targeted agents have advanced to phase III clinical trials in combination with radiation in HNSCC [7].

The early promise of cetuximab in combination with radiation in HNSCC [8] was not strengthened by a subsequent randomized trial that included cisplatin [9] that did not improve outcome. However, the drug remains important for locally advanced elderly patients who cannot tolerate cisplatin and in recurrent or metastatic disease [10]. Resistance to cetuximab has been associated with a dysregulation of normal EGFR recycling followed by increased human epidermal growth factor receptor 2 and 3 (HER2 and HER3) dimerization [11], the presence of the EGFR variant 3 (EGFRvIII) truncation mutation [12] and the presence of KRAS mutations [13]. The cooperation and signaling redundancy that exists between members of the HER family has been shown to maintain the activity of common downstream pathways despite inhibition of EGFR by cetuximab. In this study, we have used dacomitinib which is an orally active, second generation, highly selective, small-molecule pan-HER inhibitor [14] that has shown superiority over cetuximab in inhibiting growth of HNSCC cell lines [15] and has demonstrated additivity in combination with radiation with no apparent additional toxicity in a HNSCC xenograft model [16]. In a phase II study of dacomitinib in platinum-refractory recurrent or metastatic HNSCC, 10 patients (21%...
inhibitor of PI3K, tolisib (PF-05212384, PKI-587) which is a highly potent dual nation with radiation [22,27,28]. In this study we have used geda-report the efficacy of the combination of PI3K inhibitors in combi-
PI3K/MTOR inhibitors in various experimental settings in HNSCC [18,20,21]. Several studies have investigated the utility of different PI3K/MTOR inhibitors in various experimental settings in HNSCC both in vitro and in vivo [22–28]. However, only a few studies report the efficacy of the combination of PI3K inhibitors in combi-
formation as important determinant of radiosensitivity in HNSCC [18,20,21]. The lack of success of studies tar-
xing individual genes is likely due to the complexity of the sig-
aling pathways in cancer where there are multiple nodes,
leaving many variables. It would be more effective [18,19]. It has been reported that activation of downstream survival pathways leads to resistance to EGFR inhibitors. Of these downstream path-
ways, deregulation of PI3K/AKT/MTOR signaling has been identi-
fied as important determinant of radiosensitivity in HNSCC [18,20,21]. Several studies have investigated the utility of different PI3K/MTOR inhibitors in various experimental settings in HNSCC both in vitro and in vivo [22–28]. However, only a few studies report the efficacy of the combination of PI3K inhibitors in combi-

2. Materials and methods

2.1. Cell lines and drugs

The UT-SCC cell lines were provided by Dr. Reidar Grénman (Turku University Hospital, Turku, Finland). All are from a series of HPV-negative cell lines which were developed from primary and recurrent HNSCC specimens during the 1990s [30,31]. These cell lines have been maintained at low passage number (<20) and have been used these cell lines extensively in a number of studies where the cell lines, and xenografts obtained from them, have maintained consistent and reproducible characteristics [32–43]. In addition, other researchers have utilized these cell lines [44–50] and shown similar radiation responses and other characteristics to those reported in our studies. Cells were cultured and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and strepto-
mycin (100 mg/ml) and maintained at low passage number. Dacomitinib and gedatolisib was kindly provided by Pfizer (NY, USA). A 10 mM solution of each was prepared in dimethyl sulfo-
ide and stored at −70 °C for in vitro experiments.

2.2. Irradiation

Cells were irradiated with an Xstrahl X-ray System, Model RS225 (Xstrahl, UK) at a dose rate of 0.29 Gy/min, tube voltage of 160 kVp, current of 4 mA and filtration with 0.5 mAl and 0.5 mM Cu. Cells were irradiated (0.5–4 Gy) in 25 cm2 flasks at 37 °C. Animals were irradiated with a Faxitron Cabinet X-ray Sys-
tem, Model 43855F (Faxitron X-Ray, Wheeling, IL, USA) at a dose rate of 0.69 Gy/min, tube voltage of 160 kVp and current of 4 mA.

2.3. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

For the MTT assay, cells were plated into 96 well plates and allowed to attach overnight. The following day, media were exchanged for media containing various concentrations of dacom-
tinib or gedatolisib and the plates returned to the incubator. Con-
trol cultures contained media with appropriate DMSO con-
centration. After an additional 3 days, MTT (5 mg/ml PBS) was added to each well and the plate returned to the CO2 incubator for 5 h. The media/MTT was then aspirated from the wells and DMSO was added to dissolve the purple formazan. After 5 min incubation at 37 °C, absorbance readings (at 560 nm and 670 nm) were taken on a Versamax multiplate reader (Molecular Devices, Sunnyvale, CA).

2.4. Clonogenic survival assay

Cells were plated into T25 flasks at different dilutions depend-
ing on the dose of radiation such that a significant number of colo-
nies would be scored at the end of the experiment. The timings of drug administration relative to radiation were determined using the MTT assay (data not shown) to establish the optimal schedul-
ing. At 2 h prior to irradiation, media was exchanged for media containing 5 nM Gedatolisib. At 1 h prior to irradiation, media was exchanged for media containing 7.5 nM Dacomitinib. Control cells had media exchanged for normal media containing the appropriate DMSO concentration. Cells were then irradiated (0–4 Gy) and plated into flasks that contained the same media/drug that the cells had been in. Colonies were allowed to develop for 10–14 days. The colonies (~50 cells) were then stained with crystal violet, counted and surviving fractions were calculated. Data was normalized for plating efficiency and survival curves were fitted using the linear-quadratic equation.

2.5. Flow cytometric analysis of gamma-H2AX (γH2AX) and cell cycle

Cells were plated into T25 flasks and allowed to grow for 3 days. One hour prior to radiation treatment, media was exchanged for media +10 nM Dacomitinib (37 °C) or media only with DMSO (37 °C) and returned to the incubator. For γH2AX assessment, after one hour, media was exchanged for media containing 2 Gy at 37 °C and then returned to the incubator. Cells were collected at 0, 1, 2, 4, 6 and 24 h post irradi-
ation. Cells were trypsinized, washed twice with PBS and then fixed with 70% ETOH. Cell cycle analysis was studied without the addition of radiation. Samples were stored at −20 °C until analysis. For γH2AX analysis, fixed cells were pelleted and then perme-
abilized with 1% Triton X-100. Cells were washed with PBS + Tween 20(PBT) and blocked for one hour with PBT + 3% BSA. After blocking, cells were washed 2 times with PBT and then incubated with Anti-Phospho-Histone H2AX antibody (clo-
neJBW301) (Millipore Sigma, Burlington, MA) for 1 h. After incuba-
tion, cells washed 2 times with PBT and then incubated with secondary antibody, Goat anti-mouse IgG1-Alexa Fluor 488(Invi-
rogen, Carlsbad, CA), for 1 h. Cells were washed 3 times and resus-
pended in PBS. Flow cytometric analysis was performed on FACS Canto II (BD Biosciences, San Jose, CA). The mean fluorescence val-
es were recorded for the total cell population in each sample. The data was calculated by normalizing the treated samples to the mean fluorescence of the control samples.

For cell cycle analysis 106 cells were resuspended in 1 ml of PBT containing 1 mg/ml RNase A (Millipore Sigma) and 10 μg/ml pro-
pidium iodide (Millipore Sigma). Flow cytometric analysis was per-
fomed on FACS Canto II (BD Biosciences, San Jose, CA) and cell cycle analysis performed using ModFitLT (Verity Software House, Topsham, ME).

2.6. Xenograft growth delay assay

All animal experiments were approved by the Institute Animal Care and Use Committee. Xenografts were established in 4–6 week old female nude NIH III mice (Charles Rivers Laboratories, Wilming-
ton, MA) by injecting UT-SCC-14 subcutaneously into the flank, at a density of 2×106 cells per 100 μl Matrigel (Corning, Corning, NY) or UT-SCC-15 at a density of 4 × 106 per 100 μl Matrigel.
Tumor volume was measured twice weekly by digital calipers and calculated using the formula \((\pi ab^2)/6\), where \(a\) = largest diameter, \(b\) = smallest diameter. Mice were randomly assigned to experimental groups once the tumors reached a volume of 200–300 mm\(^3\). Experimental endpoint is tumor volume of 2000 mm\(^3\) or 160 days post end of treatment.

The UT-14-SCC xenograft assay consisted of eight treatment groups: (1) control, no drugs, no RT, (2) RT delivered as 2.0 Gy/day (5 times/week) for three weeks, (3) dacomitinib (10 mg/kg), oral gavage daily (5 times/week) for 3 weeks, (4) dacomitinib + RT, dacomitinib by oral gavage, 1 h prior to RT (5 times/week) for 3 weeks, (5) gedatolisib (6 mg/kg), i.v., 2 times/week (M&Th) for 3 weeks, (6) gedatolisib + RT, gedatolisib, i.v., 2 h prior to radiation 2 times/week (M&Th), (7) dacomitinib (as N group 3) + gedatolisib (as in group 5), gedatolisib administered 1 h prior to dacomitinib on M and Th, dacomitinib only T, W and F for 3 weeks, (8) dacomitinib + gedatolisib + RT. The vehicle for dacomitinib was 5% dextrose, 0.25% lactic acid solution in a volume of 0.5% methylcellulose and the volume was 0.1 cc. The vehicle for gedatolisib was 5% dextrose, 0.25% lactic acid solution in a volume of 0.15 cc.

For UT-15-SCC xenografts, the treatment groups and drug concentrations were the same but the radiation dose per fraction was increased to 3 Gy as the UT-15-SCC cells are more radioresistant than the UT-14-SCC.

2.7. Western immunoblot assay

Protein expression was analyzed in xenograft tumor tissue after treatment. Two mice from each treatment arm and each tumor model were sacrificed at the end of the three week treatment period and snap-frozen. Cellular protein was extracted from the frozen material with a SDS protein lysis buffer and stored at –20 °C. For the extraction of protein, a small piece of tumor was homogenized in T-PER (ThermoFisher-Scientific, Waltham, MA) with Halt protease/phosphatase inhibitor (ThermoFisher-Scientific, Waltham, MA) added. Unsolubilized tissue was centrifuged out and the supernatant was collected and stored at –70 °C. Equal amounts of protein (20 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane by electroblotting. After blocking, the membrane was incubated with primary antibody overnight at 4 °C. The following primary antibodies were obtained from Cell Signaling Technology (Danvers, MA), pan AKT (clone 11E7 catalog #46855), Phospho-AKT (Ser473) (clone D25E6 catalog #130385), P3 Kinase p110α (clone D5585 catalog #5405), Phospho-P3 Kinase p85(Tyr458)/p55(Tyr199) (catalog #42285), 4E-BP1 (clone 53H11 catalog #9644), Phospho-4E-BP1 (Thr37/46) (clone 236B4 catalog # 2855), S6 Ribosomal Protein (clone 54D2 catalog # 2217), Phospho-S6 Ribosomal Protein (Ser240/244) (clone D68F8 catalog # 5364), p44/42 MAPK (clone 137F5 catalog # 4695), Phospho-p44/p42 MAPK (Thr202/ Tyr204) (clone 197G2 catalog # 4377), EGF Receptor (clone D3881 catalog # 54359), and Phospho-EGF Receptor(Tyr992) (catalog # 2235) (Actin (MP Biomedicals, Irvine, CA). The membrane was washed and the secondary antibody (IRDye 800CW, Licor, Lincoln, NB) was applied for 1 h at room temperature. The membranes were analyzed with an Odyssey infra-red imaging system (Li-Cor, Lincoln, NB). Absorbance data was normalized to the actin band for each gel and then calculated as a fold change compared to untreated controls.

2.8. Statistical analysis

In vitro experiments were repeated three times and statistical analysis was carried out using a two-way t-test or one-way analysis of variance. Data are presented as the mean ± SE. A probability level of a p-value of < 0.05 was considered significant. In vivo growth delay data was analyzed based on a time-to-event analysis, i.e. the time to reach 3 times initial volume. Differences between treatment groups were analyzed using a one-way ANOVA and a Tukey post hoc test was then performed between each group comparison, p < 0.05 was considered statistically significant. Animals sacrificed prior to reaching tumor volume endpoint due to predetermined animal welfare criteria (as per protocol) were censored at the time of euthanasia.

3. Results

3.1. Dacomitinib is a potent inhibitor in EGFR overexpressing cell lines

Dacomitinib showed potent inhibition of the UT-SCC-14 cell line with an IC\(_{50}\) of 0.0023 μM (Fig. 1B). This cell line has a high level of EGFR overexpression (Fig. 1A).

A pattern of sensitivity emerged showing an inverse correlation with EGFR overexpression for three other cell lines UT-SCC-15 (IC\(_{50}\) of 0.72 μM), UT-SCC-16 (IC\(_{50}\) of 0.48 μM) and UT-SCC24A (IC\(_{50}\) of 0.16 μM) (Fig. 1A + B).

3.2. Gedatolisib is effective irrespective of EGFR status

Two cell lines at the different ends of the dacomitinib activity spectrum, UT-SCC-14 and UT-SCC-15, were selected and exposed to different concentrations of gedatolisib (Fig. 1C). The drug had similar activity in both cell lines with an IC\(_{50}\) of 0.0062 μM in UT-SCC-14 and IC\(_{50}\) of 0.019 μM in UT-SCC-15.

3.3. Neither drug modifies the radiation response in vitro

Concentrations of dacomitinib and gedatolisib that produced significant growth inhibition were combined with graded doses of radiation both alone and in combination. There was no effect of the drugs on the radiation response in UT-SCC-14 (Fig. 2A) whilst in UT-SCC-15 there was a trend for dacomitinib, with or without gedatolisib, to be radioprotective although this was not significant (Fig. 2B).

3.4. γ-H2AX staining and cell cycle analysis

To further study the observations obtained in the clonogenic assays, UT-SCC-14 and UT-SCC-15 cell lines were irradiated with 2 Gy with or without 10 nm dacomitinib and assayed for γ-H2AX staining using flow cytometry (Fig. 2C and D). The cell lines differ in their radiosensitivity in vitro (Fig. 2A and B). These differences were also apparent in Fig. 2C and D where the γ-H2AX fluorescent staining intensity was greater in UT-SCC-14 signifying more DNA double strand breaks (DSB) that peaked at 2 h with evidence of unrepaired DSBs at 24 h. In contrast UT-SCC-15 showed a modest increase in staining and was completely resolved at 24 h. Dacomitinib had only a minor effect on the radiation-induced γ-H2AX response in UT-SCC-14 but repressed the response in UT-SCC-15 (Fig. 3D).

Dacomitinib had a more profound effect on the cell cycle of UT-SCC-14 compared to UT-SCC-15 (Fig. 2E and F). There was little change in cell cycle distribution during the first 6 h but by 24 h there was an accumulation in G1 and reduction in S-phase which was more pronounced in UT-SCC-14 cells.

3.5. Effect of drugs and radiation on tumor growth and survival

The effect of the drugs and radiation are shown in Fig. 3. For clarity the data is shown for each drug and their combination as Fig. 3.
separate growth curves such that the control and radiation alone arm is repeated in each graph. The growth delay data is presented in Table 1 as the time to reach three times the initial volume at the start of treatment; each mouse has been normalized to its individual starting volume. The mean starting volume ranged from 285 mm$^3$ to 364 mm$^3$ across the eight treatment groups in UT-SCC-14 and from 162 mm$^3$ to 242 mm$^3$ in the UT-SCC-15 treatment groups.

Taking each agent into consideration, gedatolisib was ineffective in UT-SCC-14 xenografts both as a single agent and in combination with RT (Fig. 3B, Tables 1 and 2). In contrast this agent resulted in significant growth inhibition in the UT-SCC-15 xenograft (p = 0.0395) (Fig. 3E) but appeared to reduce the effect of radiation when the two modalities were combined. Radiation caused a growth delay of 132.2 days whilst this was reduced to 100.3 days in the presence of gedatolisib (p = 0.005).
Dacomitinib was active in both xenograft models causing significant growth delay as a single agent (Fig. 3A and D, Tables 1 and 2). When combined with radiation, there was significant prolongation of growth delay in the UT-SCC-14 model compared to RT alone (Table 2) and 3 of 5 animals had no detectable tumor at the end of the observation period of 160 days. In the more slowly growing UT-SCC-15 model, none of the animals treated with dacomitinib and RT had reached sacrifice criteria at 200 days.

When the two agents were used in combination with or without RT, their combined effects mirrored the effect of each agent alone (Fig. 3C and E) and their combination with radiation produced no further growth delay than seen with dacomitinib and RT alone.

3.6. Immunoblotting

A separate subset of mice was sacrificed at the end of the three week treatment period to assess the status of key proteins involved in the pathways targeted by the drugs. Fig. 4A and B shows the blots obtained for all combinations of treatments on the expression of the two of the major proteins in the EGFR/RAS/RAF/MAPK pathway whilst Fig. 5A and B shows the blots obtained on the expression major proteins involved in the AKT/PI3K/MTOR pathway. Quantitative data for phosphoprotein expression is presented in Supplemental Table 1.

Dacomitinib was active in both xenograft models causing significant growth delay as a single agent (Fig. 3A and D, Tables 1 and 2). When combined with radiation, there was significant prolongation of growth delay in the UT-SCC-14 model compared to RT alone (Table 2) and 3 of 5 animals had no detectable tumor at the end of the observation period of 160 days. In the more slowly growing UT-SCC-15 model, none of the animals treated with dacomitinib and RT had reached sacrifice criteria at 200 days.

When the two agents were used in combination with or without RT, their combined effects mirrored the effect of each agent alone (Fig. 3C and E) and their combination with radiation produced no further growth delay than seen with dacomitinib and radiation without gedatolisib.

### Table 1

| Treatment       | Time to reach 3 times pre-treatment volume (days) |
|-----------------|-------------------------------------------------|
|                 | UT-SCC-14                      | UT-SCC-15                      |
| Control         | 9.0 ± 1.7                      | 33.3 ± 5.8                     |
| DAC             | 38.8 ± 1.5                     | 87.7 ± 9.9                     |
| GED             | 16.3 ± 2.0                     | 70.4 ± 10.6                    |
| DAC + GED       | 37.5 ± 4.5                     | 96.0 ± 6.5                     |
| RT              | 59.0 ± 2.6                     | 132.2 ± 4.2                    |
| DAC + RT        | 137.9 ± 15.3                   | 156.0 ± 13.2                   |
| GED + RT        | 64.4 ± 5.2                     | 100.3 ± 4.6                    |
| DAC + GED + RT  | 139.8 ± 9.2                    | 176.4 ± 17.4                   |

### Table 2

| Treatment              | Time to reach 3 times pre-treatment volume (days) |
|------------------------|-------------------------------------------------|
|                        | UT-SCC-14                      | UT-SCC-15                      |
| Control vs. DAC only   | 0.0003                          | 0.0027                          |
| Control vs. GED only   | 0.0864                          | 0.0395                          |
| Control vs. combo      | 0.0215                          | 0.0011                          |
| Control vs. RT only    | 0.0066                          | >0.0001                         |
| Control vs. RT + DAM   | 0.0020                          | 0.0029                          |
| Control vs. RT + GED   | 0.0006                          | >0.0001                         |
| Control vs. RT + combo | 0.0050                          | 0.0013                          |
| DAC only vs. GED only  | 0.0041                          | 0.3099                          |
| DAC only vs. combo     | 0.0346                          | 0.5479                          |
| GED only vs. combo     | 0.0765                          | 0.1366                          |
| Combo vs. RT only      | 0.0231                          | 0.0159                          |
| RT only vs. DAC + RT   | 0.0106                          | 0.2707                          |
| RT only vs. GED + RT   | 0.3229                          | 0.0050                          |
| RT only vs. combo + RT | 0.0119                          | 0.0986                          |
| DAC + RT vs. GED + RT  | 0.0181                          | 0.0438                          |
| DAC + RT vs. combo + RT| 0.0153                          | 0.4740                          |
| GED + RT vs. combo + RT| 0.0148                          | 0.0188                          |
Dacomitinib was without effect on PI3K/AKT/MTOR signaling except for a significant reduction in p4EBP1 in the UT-SCC-14 tumor (Fig. 5A and B); its combination with RT was equally ineffective. Gedatolisib downregulated phosphorylation of proteins downstream of PI3K in both tumor models but the effect was more pronounced in UT-SCC-15 (Fig. 5A and B, Table 3). Combining gedatolisib with RT did not further enhance the downregulation of these phosphoproteins. The combination of all three agents was not any more effective than the gedatolisib alone at downregulating PI3K/AKT/MTOR pathway phosphoproteins. Radiation alone had minor effects on the phosphorylated proteins.

4. Discussion

Many studies have provided compelling pre-clinical evidence for the use of EGFR and PI3K pathway inhibitors to treat HNSCC [25,28,51–57]. Several of these studies have explored the combination of agents targeting both pathways with success in vitro [25,28,51,53]. However, very few studies have investigated dual targeting of these pathways in combination with radiation which remains standard-of-care in primary HNSCC. In a previous study we reported the combination of a MEK1/2 inhibitor (binimetinib) with a pan-PI3K inhibitor (buparlisib) and fractionated radiation in the same tumor models employed in this present study [42]. There were two unexpected findings from this previous study. First, the data showed discordance between the in vitro and in vivo data. Whereas buparlisib was equally effective in reducing growth of both cell lines in vitro, it had a much greater effect on UT-SCC-15 tumors while having little influence on the growth of UT-SCC-14. This was even more apparent with binimetinib which demonstrated high sensitivity against UT-SCC-14 cells in vitro compared to UT-SCC-15 but the in vivo data showed the opposite response. The second unexpected finding was that no significant benefit was gained by the combined use of the two agents with RT even though each was efficacious when used alone with radiation [42].

In this present study we obtained somewhat similar results. Again the in vitro data did not translate into the same effect in vivo. The UT-14-SCC cell line was exquisitely sensitive to dacomitinib with a 200-fold difference in response compared to UT-SCC-15 cells, yet both tumor models were responsive in vivo either with dacomitinib alone or in combination with RT. Gedatolisib showed a modest inhibition of growth in both tumor models.

Considering that both cell lines were very sensitive to gedatolisib in vitro (Fig. 1C), the in vivo effect was very disappointing (Fig. 3, Table 1). Although there was a significant downregulation of phosphorylation of PI3K/AKT/MTOR signaling proteins (Fig. 5) in both tumor models, the impact on growth was modest and there was no positive interaction with dacomitinib or RT. PI3K and MTOR both belong to the PI3K-related kinases (PIKK) superfamily and share structural domains which has led to the development of agents such as gedatolisib that target both kinases [58]. Dual inhibitors of PI3K and mTOR target the active sites of both kinases, inhibiting the pathway both upstream and downstream of AKT, thus avoiding the problem of AKT activation following ablation of the mTORC1–S6K–IRS1 negative feedback loop [59]. Preclinical in vitro cell screenings with dual PI3K/MTOR inhibitors have suggested a broader efficacy across more genotypes compared with agents targeting only one component of the pathway [60,61]. Gedatolisib inhibits PI3Kα, PI3Kγ and MTOR and we have previously studied PF-04691502 which is an ATP-competitive PI3K(α/β/δ/γ)/MTOR dual inhibitor [37] and buparlisib [42], a specific pan-class I PI3K family inhibitor, in the same tumor models. PF-04691502 and buparlisib also reduced phosphorylation of key components of the PI3K/AKT/MTOR signaling pathway in both tumor models. When comparing the three agents, buparlisib alone was the most active agent in the UT-SCC-15 tumor model followed by gedatolisib whereas PF-04691502 was inactive. In the UT-SCC-14 tumor model, buparlisib was inactive whereas the two dual PI3K/MTOR inhibitors showed modest activity at the doses used. In the context of radiation treatment only PF-04691502 [37] was able to significantly enhance the radiation response in UT-SCC-14.
whilst buparlisib [42] was the only active radiation enhancer in UT-SCC-15. In this present study gedatolisib appeared to diminish the radiation effect in this tumor model (Fig. 3, Table 1). To an extent, the growth delay data mirrored the pathway inhibition analysis where our previous research showed that PF-04691502 was very effective agent at reducing phosphorylation AKT, S6 and 4EBP1 in UT-SCC-14 in combination with radiation [37] whilst this present study demonstrated that gedatolisib was the least effective agent at inhibiting the PI3K pathway during radiation treatment.

Our results clearly demonstrate the complexity of targeting the PI3K/AKT/MTOR pathway as this pathway is activated in cancers via several different mechanisms. These include amplification or mutational activation of genes encoding receptor tyrosine kinases, RAS, and/or the p110α catalytic subunit of PI3K (PIK3CA) and inactivation of the tumor suppressor gene, PTEN. In head and neck cancer, alteration in genes such as PTEN, TSC1 and PIK3CA encompass over 30% of the mutations found in this cancer [62–64]. In addition, loss of p53 function promotes mTORC1 activation and regulation of PTEN transcription [65]. In a previous study we have demonstrated that the UT-SCC-15 cell line harbored more variants in AKT1, AKT2, MTOR, PIK3CA, PIK3R1, PTEN and TP53 than the UT-SCC-14 cell line [37].

The results with dacomitinib were impressive and encouraging. Both tumor models, despite their different spectrum of mutations

Fig. 5. Immunoblotting for each drug and combination with radiation in UT-SCC-14 xenografts (A) and UT-SCC-15 xenografts (B) for total and phosphorylated proteins in the PI3K pathway. Actin controls are shown for each gel (data not shown for clarity). There was n = 2 for these studies.
Table 3
Quantitative analysis of immunoblotting data. The data was normalized to each actin control and then to the untreated control animal data and expressed as mean fold change.

|                  | pEGFR | pMAPK | pPI3K | pAKT | pMTOR | p4EBP1 | pS6 |
|------------------|-------|-------|-------|------|-------|--------|-----|
| UT-SCC-14        |       |       |       |      |       |        |     |
| DAC              | 0.12  | 0.57  | 0.82  | 0.80 | 0.74  | 0.22   | 1.01|
| GED              | 0.74  | 1.11  | 0.89  | 0.54 | 0.62  | 0.75   | 0.41|
| DAC/GED          | 0.40  | 0.84  | 1.06  | 0.48 | 0.93  | 1.02   | 0.88|
| RT               | 0.62  | 1.59  | 0.87  | 1.21 | 1.071 | 0.90   | 1.12|
| DAC/RT           | 0.03  | 0.62  | 0.59  | 0.42 | 0.92  | 0.27   | 0.87|
| GED/RT           | 0.47  | 1.52  | 0.78  | 0.68 | 0.83  | 0.83   | 0.80|
| DAC/GED/RT       | 0.029 | 0.512 | 0.72  | 0.29 | 0.48  | 0.58   | 0.16|
| UT-SCC-15        |       |       |       |      |       |        |     |
| DAC              | 0.17  | 0.36  | 1.01  | 0.36 | 1.18  | 0.92   | 0.33|
| GED              | 1.32  | 0.64  | 0.91  | 0.45 | 0.43  | 0.52   | 0.39|
| DAC/GED          | 0.41  | 0.47  | 1.067 | 1.167| 0.64  | 0.67   | 0.52|
| RT               | 1.2   | 0.79  | 0.84  | 1.09 | 0.54  | 1.03   | 1.02|
| DAC/RT           | 0.05  | 0.35  | 0.73  | 1.06 | 1.03  | 1.82   | 0.41|
| GED/RT           | 0.47  | 0.61  | 1.02  | 1.11 | 0.47  | 0.70   | 0.65|
| DAC/GED/RT       | 0.28  | 0.47  | 0.75  | 0.61 | 0.59  | 0.57   | 0.31|

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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