Dual blockade of PI3K and MEK in combination with radiation in head and neck cancer

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Background and purpose: In this study we have combined fractionated radiation treatment (RT) with two molecular targeted agents active against key deregulated signaling pathways in head and neck cancer. Materials and methods: We used two molecularly characterized, low passage HNSCC cell lines of differing biological characteristics to study the effects of binimetinib and buparlisib in combination with radiation in vitro and in vivo. Results: Buparlisib was active against both cell lines in vitro whereas binimetinib was more toxic to UT-SCC-14. Neither agent modified radiation sensitivity in vitro. Buparlisib significantly inhibited growth of UT-SCC-15 alone or in combination with RT but was ineffective in UT-SCC-14. Binimetinib did cause a significant delay with RT in UT-SCC-14 and it significantly reduced growth of the UT-SCC-15 tumors both alone and with RT. The tri-modality treatment was not as effective as RT with a single effective agent. Conclusions: No significant benefit was gained by the combined use of the two agents with RT even though each was efficacious when used alone.

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Background and significance

HPV-negative head and neck cancer (HNSCC) carries a poor prognosis despite optimal treatment with chemoradiation [1] which approaches the limit of tolerance. It is likely that improvements will be made by novel therapies based on the molecular mechanisms of HNSCC. Early success was achieved by targeting the EGFR pathway [2] but since then very few targeted agents have advanced to phase III trials with radiotherapy [3].

Genomic analysis of HNSCC from the TCGA proposed at least 15 significantly mutated genes including CDKN2A, TP53, PIK3CA, FAT1, MLH2, TGFB2, HLA-A, NOTCH1, HRAS, NFE2L2, and CASP8 [4]. Many of the genes converge on the EGFR/RAS/RAF/PI3K/ AKT/mTOR signaling cascade which has been reported to be one of the most frequently altered signaling pathways in HNSCC [5]. These pathways offer multiple targets for therapy. However, targeting these pathways with single agents has produced little success in translating this approach into clinical benefit [6].

This lack of success is likely due to the complexity of the signaling pathways in cancer where there are multiple nodes, feedback loops, crosstalk and redundancy. To overcome these issues it would seem rational that targeting these pathways at several points simultaneously might be more effective [7,8]. One of the mechanisms of cetuximab resistance is the presence of mutations, in particular RAS, that constitutively activate key downstream signaling mediators [9]. In addition, reports have also suggested that BRAF may also be frequently mutated in HNSCC [10]. This has led us and others [8] to target the EGFR signaling downstream at the level of MEK1 and 2. Another key pathway identified by the TCGA study was PI3K/AKT/mTOR signaling which has been shown to have an important role in the pathogenesis of HNSCC [11] and there is evidence PI3K antagonists are active HNSCC cells [12,13].

In this study, we investigated binimetinib, a potent oral inhibitor of MEK1/2 currently being studied in several clinical trials [14–16], and buparlisib, a specific oral inhibitor of the pan-class I PI3K family also under investigation in clinical trials [17–19] in two contrasting HNSCC models with different EGRF, HRAS and PI3K status and studied their combination with fractionated radiation.
Materials and methods

Cell lines and drugs

The UT-SCC-14 and UT-SCC-15 cell lines were provided by Dr. Reidar Grénman (Turku University Hospital, Turku, Finland). Both are low passage HPV-negative cell lines and their culture and molecular characterization have been previously described [20]. Buparlisib and binimetinib were kindly provided by Novartis Pharma (Basel, Switzerland). A 10 mM solution of each was prepared in dimethyl sulfoxide and stored at −70 °C for in vitro experiments.

Irradiations

Cells were irradiated as previously described with an Xstrahl X-ray System, Model RS225 (Xstrahl, UK) [20]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Three-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to assess the effects of the drugs on cell growth/viability. Cells were plated into 96 well plates and allowed to attach overnight. The next day the media was exchanged for media containing various concentrations of buparlisib and binimetinib and the plates returned to the incubator. After an additional 3 days, MTT (5 mg/mL in phosphate-buffered saline) was added to each well and the plate returned to the CO2 incubator for ~5 h. Media containing the MTT was then aspirated from the wells, and dimethyl sulfoxide 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). To assess the effect of the combination of the drugs on cell growth, different single concentrations of buparlisib (0.1, 0.25 and 0.5 μM) were incubated with increasing concentrations of binimetinib. To study timing of drug exposure in combination with 4 Gy irradiation (RT), 0.5 μM buparlisib or 0.5 μM binimetinib were added to plated cells either 1 h pre-RT or 1, 4 or 24 h post-RT.

Clonogenic assay

Cells were irradiated and then plated into flasks containing 0.2 μM buparlisib, 0.1 μM binimetinib or their combination. Untreated cells and drug(s)-only treated cells were also assessed. Colonies were allowed to develop for 10–14 days, stained with crystal violet counted, and surviving fractions calculated. Data was normalized for plating efficiency to the unirradiated, non-drug treated controls and survival curves were fitted using the LQ model.

Fig. 1. The effect buparlisib (BUP) (A) and binimetinib (BIN) (B) on the growth of UT-SCC-14 and UT-SCC-15 cells in vitro as single agents or in combination (C and D). In C and D, the concentration of buparlisib was 0.25 μM.
Immunobloting

Two tumors from each treatment group were collected at the end of treatment for protein analysis as previously described [20]. After blocking, the membrane was incubated with antibodies. All antibodies except anti-actin were obtained from Cell Signaling Technology, Danvers, MA and used at the following dilutions: p-AKT (1:2000), phospho-AKT (Ser 473) (1:2000), PI3 Kinase p110α (1:1000), phospho-PI3 Kinase p85(Tyr458)/p55(Tyr199) (1:1000), p44/42 MAPK(ERK1/2) (1:1000), phospho-p44/42 MAPK(ERK1/2) (Thr202/Tyr204) (1:1000), MEK1/2 (1:1000), phospho-MEK1/2 (Ser217/221) (1:1000), phospho-4E-BP1(Thr37/46) (1:1000), phospho-S6 Ribosomal Protein(Ser240/244) (1:1000) and actin (1:20,000; MP Biomedicals, Solon, OH). Membranes were incubated with IRDye 800CW (1:20,000; Licor, Lincoln, NB, USA) and analyzed with an Odyssey infra-red imaging system (Li-Cor). Data was analyzed as the ratio of phosphorylated protein to total protein which was calculated by normalizing each band to its actin control and then taking the ratio of these ratios.

Xenograft growth delay

After approval by the Animal Care Committee (AL-15-07), xenografts were established as subcutaneous tumors in 4-to-6-week old female nude NIH III mice (Charles Rivers Laboratories, Wilmington, MA, USA) as previously described [20]. Tumor volume was measured twice weekly by digital calipers and calculated using the formula \( \frac{ab^2}{6} \) (\( a = \) largest diameter, \( b = \) smallest diameter). When the tumors reached a volume of 200–300 mm\(^3\), animals were randomly assigned to the experimental groups. Experiment endpoint was a tumor volume of 2,000 mm\(^3\). Seven mice were used in each experimental group.

Radiation was delivered using a Faxitron Cabinet X-ray System at a dose rate of 0.69 Gy/min, tube voltage of 160 kVp, current of
4 mA and filtration with 0.5 mM Al and 0.5 mM Cu. Unanesthetized animals were restrained and protected in custom-made lead jigs with only the right flank exposed and irradiated in groups of 4 in a cross-shape configuration with the flank tumors positioned to the center of the radiation field. Buparlisib (10 mg/kg), binimetinib (5 mg/kg) in 0.5% methylcellulose or their combination was given by oral gavage 4 h post radiation treatment, 5 days/week for three weeks. Sham oral gavage was 0.5% methylcellulose only. Radiation treatment (RT) consisted of 2 Gy/day, five times/week for three weeks for UT-SCC-14. For UT-SCC-15 tumors, the dose per fraction was increased to 3 Gy due to the relative radioresistance of this cell line. For each tumor there were eight treatment groups: (1) control with sham oral gavage (2) RT and sham oral gavage (3) buparlisib (4) RT followed by buparlisib (5) binimetinib (6) RT followed by binimetinib (7) buparlisib in combination with binimetinib (8) RT followed by buparlisib and binimetinib.

**Statistical analysis**

*In vitro* experiments were repeated three times and statistical analysis was carried out using a two-way t-test or one-way analy-
Table 1
The effect of the buparlisib (BUP) and binimetinib (BIN) and radiation (RT) on growth delay of UT-SCC-14 and UT-SCC-15 xenografts. The data are expressed as mean ± S.E. M for growth delay. Significant differences in the statistical comparisons are highlighted in bold type.

|                  | UT-SCC-14  | UT-SCC-15  |
|------------------|------------|------------|
| Treatment        |            |            |
| Control          | 24.9 ± 3.6 | 28.8 ± 2.3 |
| RT only          | 63.3 ± 6.8 | 95.6 ± 5.6 |
| BUP only         | 28.5 ± 1.4 | 85.0 ± 4.4 |
| BIN only         | 28.4 ± 3.9 | 82.3 ± 7.1 |
| BUP/RT           | 36.2 ± 2.9 | 108.0 ± 8.5|
| BUP/BIN          | 64.2 ± 0.6 | 160.2 ± 9.8|
| BUP/BIN/RT       | 97.0 ± 10.6| 123.9 ± 8.3|
| BUP/BIN/RT       | 101.8 ± 11.1| 112.6 ± 9.9|
| Comparison       | p-value    | p-value    |
| con v BUP        | 0.9997     | 0.0016     |
| con v BIN        | 0.9992     | 0.0005     |
| con v BUP/BIN    | 0.8755     | >0.0001    |
| con v RT         | 0.009      | >0.0001    |
| con v BUP/RT     | 0.0007     | >0.0001    |
| con v BIN/RT     | >0.0001    | >0.0001    |
| BUP v BUP/BIN    | 0.98234    | 0.51209    |
| BIN v BUP/BIN    | 0.98067    | 0.21756    |
| RT v BUP/BIN     | 0.0539     | 0.99242    |
| RT v RT/BUP      | 1.0000     | 0.0008     |
| RT v RT/BIN      | 0.00785    | 0.40698    |
| RT v RT/BIN/RT   | 0.00175    | 0.93852    |
| RT/BUP v RT/BIP/BIN | 0.00234  | 0.01203    |
| RT/BIN v RT/BUP/BIN| 0.99925  | 0.96853    |

Timing of radiation and drug exposure

0.5 μM buparlisib or 0.5 μM binimetinib were added to plated cells 1 h pre-RT or 1, 4 or 24 h post-radiation treatment with 4 Gy (Fig. 2). There were no significant differences between 1 h pre-, 1 h post or 4 h post irradiation in either cell line whilst the 24 h post-irradiation was ineffective. In conjunction with previous experience [21], administration of drug 4 h post-irradiation was chosen for the in vivo studies.

The effect of drugs on clonogenic survival

Fig. 3 shows that there was no significant modification of the radiation response in either cell line using either concurrent or 4 h post-irradiation drug exposure. The drugs alone had no effect on clonogenic survival (data not shown).

Effects of drugs and radiation on growth delay in vivo

Fig. 4 shows the growth curves for UT-SCC-14 and UT-SCC-15 xenografts and Table 1 presents the analysis of the data. One-way analysis of variance revealed significant differences between the treatment groups (p < 0.05) whilst post hoc analysis identified several significant results (Table 1).

Buparlisib was ineffective in the UT-SCC-14 tumors either alone or in combination with radiation (Fig. 4A). In contrast, this agent significantly inhibited growth in UT-SCC-15 both alone and in combination with RT (Fig. 4D). The combination of buparlisib and RT in UT-SCC-15 showed the greatest growth delay and the combination was highly significant (p = 0.0008) compared to RT alone.

Binimetinib alone did not cause growth delay in UT-SCC-14 but did cause a significant delay in combination with RT (Fig. 4B). As with buparlisib, binimetinib significantly reduced growth of the UT-SCC-15 tumors both alone and in combination with RT (Fig. 4E).

When buparlisib and binimetinib were combined they elicited a significant growth delay compared to controls in UT-SCC-15 but not UT-SCC-14. When the combination of drugs was combined with RT, there was significant increase in growth inhibition in UT-SCC-14 but this was mainly attributed to binimetinib (Fig. 4C and Table 1). In UT-SCC-15, the RT and drugs combination diminished the overall growth delay compared to RT with either agent alone.

Alterations in the MEK/RAF pathway

Fig. 5A and B shows the effect of the different treatments on MEK and MAPK and their phosphorylated forms in UT-SCC-14 and UT-SCC-15 respectively. Fig. 7A and B shows the ratio of phosphorylated to total protein after correcting each for their loading control. The MEK/MAPK pathway showed greater constituent activation in UT-SCC-15 with a higher ratio of phosphorylated to total protein for both MEK and MAPK. The response to binimetinib highlights the influence of genomic alterations between the cell lines. UT-SCC-15 harbors mutations in KRAS and HRAS which have been shown to result in an increase in pMEK in response to MEK inhibitors through feedback-mediated RAF activation [22]. pMEK levels were increased 5-fold in binimetinib treated tumors whilst levels were further increased when binimetinib was combined with radiation. However, binimetinib was able to effectively decrease levels of pMAPK in both tumor models both alone and in combination with RT. Radiation treatment caused an increase of pMEK and pMAPK in UT-SCC-14 that wasn’t evident in UT-SCC-15 possibly due to this pathway already being active in the latter cell line. Interestingly, buparlisib treatment alone or with RT resulted in an increase in pMAPK in UT-SCC-15 but not UT-SCC-14 indicating pathway crosstalk [23].

Molecular characterization of the UT-SCC-14 and UT-SCC-15 cell lines

The cell lines have been characterized previously using next generation DNA sequencing using the Qiagen Comprehensive Cancer Panel [20]. The more radioresistant UT-SCC-15 cell line harbored mutations in both HRAS and KRAS as well as several DNA repair genes including ATM, FANCD2, MSH2 and PMS2 that were not found in UT-SCC-14 whilst UT-SCC-14 carried mutations in EGFR, ERBB4, MTOR, RB1 and TP53 that were not found in UT-SCC-15. The UT-SCC-15 cell line harbored more variants in the PI3K/AKT/MTOR pathway than the UT-SCC-14 cell line [20].

Sensitivity of cell lines to buparlisib and/or binimetinib

Both cell lines showed a similar, classical sigmoid dose response to increasing concentrations of buparlisib (Fig. 1A) with IC50 values of ~0.5 μM. In contrast, the cell lines showed differential sensitivity to binimetinib with UT-SCC-14 cells being more sensitive than UT-SCC-15 with IC50 of 0.444 μM (Fig. 1B) whilst UT-SCC-15 had a much flatter dose response curve; the IC50 was not reached at 10 μM. When the drugs were combined by adding 0.1, 0.25 or 0.5 μM buparlisib (the data for 0.25 μM is shown in Fig. 1C and D) to increasing concentrations of binimetinib, there was no significant change in cell viability when the drugs were combined.


Alterations in the PI3K/AKT/MTOR pathway

Fig. 6A and B shows the effect of the agents and their combinations on components of the PI3K/AKT/MTOR pathway UT-SCC-14 and UT-SCC-15 respectively. Fig. 7C and D shows the ratio of phosphorylated to total protein after correcting each for their loading control. Buparlisib is a pan-class I PI3K inhibitor and was effective at reducing phosphorylated PI3K and AKT, as well as the downstream signaling proteins pS6 and p4EBP1 S6 and 4EBP1 in the UT-SCC-14 tumor model (Figs. 6A and 7C). However, buparlisib failed to reduce phosphorylation of these proteins in the UT-SCC-15 tumor model (Figs. 6B and 7D). Binimetinib had no effect of UT-SCC-14 signaling but enhanced the phosphorylation of downstream signaling (S6 and 4EBP1) in UT-SCC-15 xenografts. A similar effect was observed with RT where there was little change in PI3K/AKT/MTOR signaling in UT-SCC-14 but an increase in pS6 and p4EBP1 in UT-SCC-15. Combining the drugs resulted in a similar reduction in protein phosphorylation as caused by buparlisib alone in UT-SCC-14 and did abrogate the increases pS6 and p4EBP1 caused by binimetinib alone. Combining RT with buparlisib reduced the effectiveness of buparlisib alone to inhibit the PI3K/AKT/MTOR pathway in UT-SCC-14 and tended to increase the phosphorylation of proteins in UT-SCC-15. Binimetinib and RT had little effect on signaling in either xenograft model. Interestingly, combining both drugs with RT abolished the reduction of phosphorylated proteins seen in UT-SCC-14 by buparlisib alone and with RT and again appeared to increase phosphorylation of AKT, S6 and 4EBP1 in UT-SCC-14.

Discussion

To our knowledge, this is the first study to combine two molecular targeted agents with a realistic fractionation schedule in two contrasting models of head and neck cancer. We routinely use the UT-SCC-14 (primary tongue tumor) and UT-SCC-15 (nodal recurrence of tongue tumor) cell lines based on their reproducible growth pattern (Fig. 4), but more importantly, their differing mutational landscape and radiation and drug sensitivity [20]. The more radioresistant UT-SCC-15 cell line harbors mutations in both HRAS
and KRAS as well as several DNA repair genes including ATM, FANCD2, MSH2 and PMS2 whilst UT-SCC-14 carried mutations in EGFR, ERBB4, RB1 and TP53. In addition UT-SCC-15 has more variants in AKT1, AKT2, MTOR, PIK3CA, PIK3R1, PTEN and TP53 than UT-SCC-14 [20].

The data showed some discordance between the in vitro and in vivo data. Buparlisib was equally effective in reducing growth of both cell lines in vitro (Fig. 1A) but the in vivo data revealed a much greater effect of the drug on UT-SCC-15 while having little effect on the growth of UT-SCC-14. More unexpected were the results 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 (Fig. 4B and E). A similar picture emerged when the drugs were combined. The in vitro data showed no obvious interaction of the drugs on cell growth or clonogenic survival but both xenograft models showed significant increases in growth delay when the drugs (without RT) were combined. Neither drug affected radiosensitivity in vitro although other studies have reported radiosensitizing effects of PI3K/MTOR inhibitors [24–28] and MEK inhibitors [27,29] but this has not always found to be the case [30]. Timing of administration of the two modalities may be an issue may be a crucial issue. Our data suggested that giving the drug 24 h after radiation was not effective and previous studies have shown that giving PI3K/MTOR inhibitors 24 h before radiation was also ineffective [31]. We found no profound differences between 1 h prior or 1 and 4 h post radiation (Fig. 2) and based our scheduling on this and previous data where we demonstrated that radiation upregulates PI3K, AKT and MTOR levels [20]. Further studies are needed to establish the best scheduling in vivo.

The drug data in isolation indicate a complex interplay between the EGFR/RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways. These
pathways extensively cross-talk [23] to both positively and negatively regulate each other that is dependent on the activation status of key genes such as **KRAS, RAF, PTEN and PI3KCA**. Binimetinib was equally effective at reducing levels of the key signaling protein pMAPK in both xenograft models but did not affect growth in UT-SCC-14. Interestingly, buparlisib was effective at reducing phosphorylation of all components of the PI3K pathway in UT-SCC-14 but that didn’t translate into a significant growth delay whereas phosphorylation of PI3K and AKT levels were not changed in UT-SCC-15 but a significant growth inhibition was observed. However, despite no change in phosphorylated PI3K and AKT, downstream phosphorylation of S6 and 4EBP1 was reduced. When the drugs were combined in both tumor models, the reduction in levels of the key phosphoproteins appeared no greater than that that produced by the active drug alone yet a highly significant growth inhibition was seen in UT-SCC-15 where the effect was similar to the 15 fractions of 3 Gy; no effect was seen in UT-SCC-14. growth inhibition was enhanced in both xenografts.

**Fig. 7.** The ratio of phosphorylated to total protein for each protein studied. In A (UT-SCC-14) and B (UT-SCC-15) the data is presented for MEK ( ) and MAPK ( ). In C (UT-SCC-14) and D (UT-SCC-15) the data is presented for PI3K ( ), AKT ( ), S6 ( ) and 4EBP1 ( ). Each bar represents the average of the two samples per treatment.
The addition of radiation adds another level of complexity as the radiation-induced DNA damage response can activate multiple signaling pathways within cells [32]. There are several reports of radiation activation of the EGFR/RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways in cell lines [33–36] including our previous study [21]. The ability of radiation to activate pathways is likely to depend on multiple influences and will vary from one cell type to another. This was the case in this study where radiation caused greater activation of the EGFR/RAS/RAF/MEK pathway in UT-SCC-14 which overexpresses EGFR than was found in UT-SCC-15 which harbors a mutation in HRAS. As a result binimetinib was much more effective in downregulating pMAPK in UT-SCC-15 in combination with radiation than it was in UT-SCC-14. The UT-SCC-15 cell line carries a heavier burden of variants associated with the PI3K/AKT/mTOR pathway [21] but neither cell line have activating mutations in PIK3CA which has been shown previously to confer greater sensitivity to PI3K or PI3K/mTOR inhibitors [37,38]. Both cell lines were equally responsive to buparlisib in terms of inhibition of phosphorylation of the key signaling proteins but buparlisib was not as effective in the UT-SCC-14 model in the combination with radiation as it was in the UT-SCC-15. It was also interesting to note that although pMAPK wasn’t suppressed when binimetinib was combined with RT, it did still result in a significant growth inhibition compared to RT alone. One reason maybe that binimetinib appeared to increase expression of the downstream proteins, S6 and 4EBP1, in the PI3K/AKT/MTOR pathway. These data add further evidence that the effectiveness of the agents is affected by crosstalk between the EGFR/RAS/RAF/MEK and PI3K/AKT/mTOR pathways as has been shown in other studies [9,23–39,41].

We have focused on the interplay between the EGFR/RAS/RAF/MEK and PI3K/AKT/mTOR signaling pathways and relevant genomic changes in the models in this study but it is important to consider that other downstream consequences of alterations in these pathways are likely to have contributed to the differential response to radiation and the targeted agents. There is some evidence linking PI3K/AKT/mTOR pathway to the DNA damage response (DDR) [44]. Suppression of PI3K has been demonstrated to sensitize breast cancer cells to poly ADP ribose polymerase (PARP) inhibitors by downregulating homologous recombination (HR) [45] and inhibition of the PI3K/AKT/mTOR pathway sensitized endometrial cells to PARP inhibitors [46]. Conversely, inhibition of the EGFR signaling pathway has previously been shown not to induce radiosensitization in the cell lines used in the study [47]. The UT-SCC-15 cell line and xenograft is more radioresistant than UT-SCC-14 [48]. Further studies are required to investigate the interplay between the DDR and the combination therapy used in this study. Another mechanism that may help to explain the difference between the in vitro and in vivo data may involve modulation of tumor vasculature by an effect on endothelial cells. It has been demonstrated that dual inhibitor of PI3K and MTOR can improve tumor oxygenation and vascular structure over a prolonged period and block VEGF phosphorylation in human umbilical venous endothelial cells and microvascular dermal cells [24,49]; this effect would only be apparent in vivo. We have shown in a previous study that differences in hypoxia and vascularization exist between these two xenograft models and that radiation has a differential effect on these physiological parameters [50].

Unexpectedly, this study demonstrated 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. Both buparlisib and binimetinib showed activity in combination with radiation and this warrants further investigation into blockade of these signaling pathways as radiosensitizing strategies. Although both tumor models were squamous cell carcinomas, they responded differently to the treatments and highlight the need to study multiple tumor models, if possible, to account for the likely heterogeneity of response of human tumors in clinical studies. In addition, this study highlights that combining radiation with molecular targeted agents against pathways which are activated by the radiation treatment is not straightforward and more research into the scheduling of the agents is required.

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Conflict of interest statement

There are no conflicts of interests for the authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ctro.2018.04.003.

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