Anti-*POMC* siRNA reduces ACTH secretion in a cell culture model of Cushing's disease

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Background

Cushing's disease is due to a pituitary corticotroph adenoma autonomously-secreting excessive amounts of adrenocorticotrophic hormone (ACTH) and is the most common cause of ACTH-dependent, endogenous Cushing's syndrome[1]. Increased ACTH secretion stimulates excess cortisol production and release into the circulation. Long-term sequalae of chronic exposure to hypercortisolaemia include physical, metabolic and psychological co-morbidities and increased standard mortality rate[2]. Transsphenoidal resection of the adenoma is currently the only curative treatment for Cushing's disease and remains the first-line treatment, unless contraindicated. However, there are long-term side effects and long-term recurrence is high, even amongst patients who achieve initial remission[3].There is a lack of suitable medical alternatives.

RNA-interference is a naturally-occurring regulatory process that causes post-transcriptional gene silencing by neutralising targeted mRNA molecules before they can be translated into protein[4]. This can be utilised to knock-down the expression of specific genes using double-stranded small interfering RNAs (siRNA).

Proopiomelanocortin (POMC) is the polypeptide precursor of ACTH. It is cleaved in the anterior pituitary gland to form ACTH, and is coded for by the *POMC* gene.

Hypothesis

We hypothesise that anti-*POMC* siRNA could be a novel effective medical treatment for Cushing's disease; post-transcriptional silencing of *POMC* expression could ultimately lower the concentration of circulating ACTH, leading to decreased stimulation of adrenal steroidogenesis.

Aim

To investigate if siRNAs directed against the *POMC* gene can cause a reduction in the levels of ACTH secreted by an *in vitro* cell culture model (AtT-20 cells) of Cushing's disease.

Methods

Cell culture model – AtT-20 (a neuroendocrine cell line of immortalised murine anterior pituitary corticotrope tumour) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX™, 10% foetal calf serum and 1% penicillin/streptomycin.

siRNA molecules - three siRNAs referred to as siRNA-1, -2 and -3 (Ambion), were designed to target different exonic regions within the *POMC* mRNA (Figure 1). Designed to have homology to both mouse and human sequences[5]. Control siRNAs were ON-TARGET*plus*™ non-targeting siRNA (Dharmacon), designed to have no known target, to distinguish the sequence-specific silencing action of anti-*POMC* siRNA on ACTH level from any non-specific effects, and "Mismatch" and "Scrambled" siRNA-3 molecules custom-designed to investigate sequence specificity.

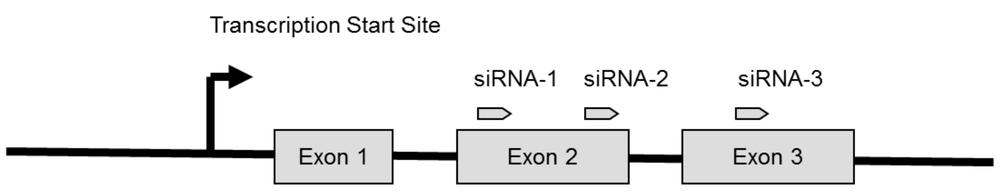
siRNA transfections - AtT-20 cells were plated in 2 ml of antibiotic-free medium into 6-well plates, at $2x10^5$ cells per well. Cells were incubated for 24 hours at 37°C in a 5% CO_2 humidified incubator. After 24 hours, the medium was removed and replaced with 1 ml of fresh antibiotic-free medium and 0.5 ml of OptiMEM per well. AtT-20 cells were transfected with each *POMC* siRNA at 30 nM using Lipofectamine™-2000 (Invitrogen) as the transfection reagent (TR). The cells were incubated for 24 hours post-transfection and the culture medium collected for ACTH assay. Transfection experiments were repeated independently 3 times.

Control treatments – These were: 1) a no-transfection-reagent control, to assess the efficiency of siRNA taken up by the cells in the absence of transfection reagent; 2) a minus-siRNA control with transfection reagent only, which allowed any toxic effects due to the Lipofectamine to be accounted for; and 3) an untreated control, with no siRNA or transfection reagent added, to measure baseline ACTH secretion by the AtT-20 cells.

ACTH measurement - An Immulite 2000 ® ACTH immunoassay (Siemens Healthcare) was used to detect the presence of ACTH in the AtT-20 cell culture supernatants. The concentration of ACTH was measured in pg/ml.

Cell viability – Trypan Blue staining was used to estimate viable cell numbers.

Figure 1: Diagrammatic representation showing siRNA-1, -2 and -3 target positions on *POMC* gene exons. siRNA-1 and -2 target exon 2 of POMC, and siRNA-3 targets exon 3.



Results

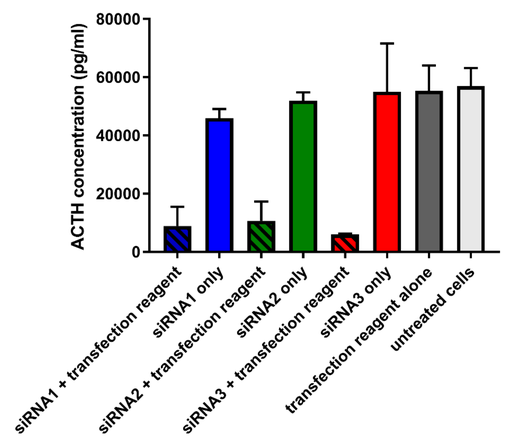


Figure 2: Effect of anti-*POMC* siRNAs on secretion of ACTH from AtT-20 cells.
The graph shows the mean (± SD) results of three independent siRNA transfection experiments on ACTH levels. Culture media was removed from wells containing AtT-20 cells 24 hours after transfection. ACTH levels in the medium were analysed by immunoassay. Controls such as transfection reagent only and no transfection were included. No treatment control provided the baseline ACTH levels secreted into culture media by the AtT-20 cells.
All 3 siRNA molecules successfully reduced ACTH levels when transfected with transfection reagent, compared to naked siRNA transfection, and siRNA-3 produced the greatest overall decrease in ACTH.

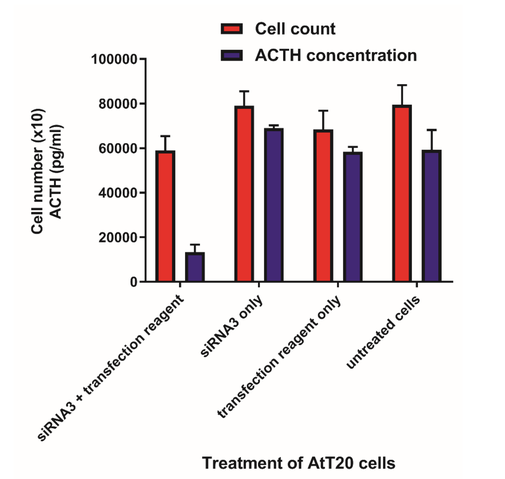


Figure 3: ACTH concentration and total number of viable AtT-20 cells following transfection with anti-*POMC* siRNA-3.
The graph displays the mean (± SD) results of three independent experiments using 30 nM of anti-*POMC* siRNA-3 to establish whether the reduction in ACTH concentration secreted by the AtT-20 cells was due to transfection with siRNA or due to cytotoxic effects of the Lipofectamine transfection reagent.
Cell viability remained similar to untreated cells when Lipofectamine was included in experiments to transfect AtT-20 cells.

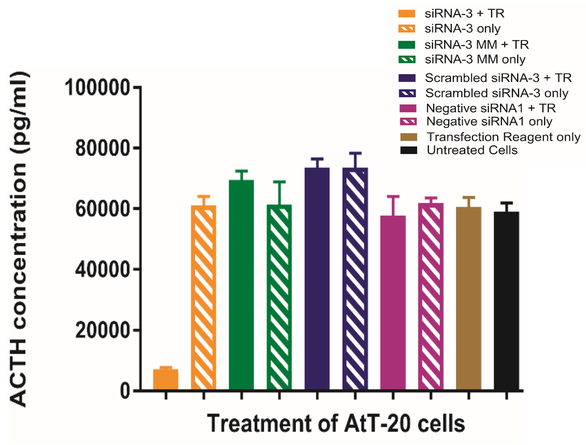


Figure 4: Effect of control siRNAs on ACTH secretion by AtT-20 cells.
siRNA-3, mismatch and scrambled siRNAs and a commercial non-targeting siRNA were transfected into AtT-20 cells with Lipofectamine®-2000 reagent (TR) or naked and ACTH concentration was measured.
Transfections with control siRNAs had no effect on ACTH secretion.

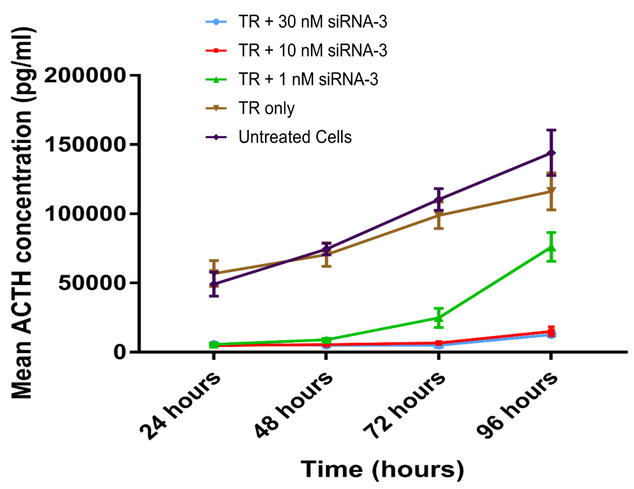


Figure 5: Dose-response effect of varying the concentration of anti-*POMC* siRNA-3 on ACTH secretion by the AtT-20 cells.
AtT-20 cells were transfected with siRNA-3 molecule at 30, 10 and 1 nM. Controls were untreated cells and cells that had been treated with Lipofectamine®-2000 (TR) only. Results display the mean ACTH concentration ± SD of 4 replicates from one experiment.
Increasing the concentration of siRNA suppressed the ACTH secretion in a dose-dependent manner.

Conclusions

- It is possible to reduce ACTH secretion using anti-*POMC* siRNAs.
- The reduction in ACTH secretion was not solely due to transfection reagent-mediated cell death.
- The decrease in ACTH is due to the specific silencing effects of anti-*POMC* siRNAs and not to non-specific silencing by double-stranded siRNA.
- Further work will investigate the effects of siRNA *POMC* mRNA using *in vivo* experiments in a mouse model.

References

1. Lacroix, A., Feelders, R.A., Stratakis, C.A., and Nieman, L.K. (2015). Cushing's syndrome. Lancet *386*, 913-927.
2. Newell-Price, J., Bertagna, X., Grossman, A.B., and Nieman, L.K. (2006). Cushing's syndrome. The Lancet *367*, 1605-1617.
3. Buliman, A., Tataranu, L.G., Paun, D.L., Mirica, A., and Dumitrache, C. (2016). Cushing's disease: a multidisciplinary overview of the clinical features, diagnosis, and treatment. Journal of Medicine and Life *9*, 12-18.
4. Daka, A., and Peer, D. (2012). RNAi-based nanomedicines for targeted personalized therapy. Advanced Drug Delivery Reviews *64*, 1508-1521.
5. Munir, A. (2013). RNA interference as therapy in a model of Cushing's disease. PhD thesis. University of Sheffield.

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