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Connectivity Mapping (ssCMap) To Predict A20 Inducing Drugs Anti-inflammatory Action In Cystic Fibrosis

B Malcomson, H Wilson, E Veglia, G Thilagayampanal, R Barsden, S Donegan, A El Banna, JS Elborn, M Ennis, C Kelly, SD Zhang, and BC Schock.

1Centre for Infection and Immunity, Queen’s University of Belfast, Belfast, BT9 7AE, UK. 2Dipartimento di Scienze e Tecnologia del Farmaco, Università di Torino, Via P. Giuria 9, 10125, Turin, Italy. 3Centre Cancer Research and Cell Biology, Queen’s University of Belfast, Belfast, BT9 7AE, UK. 4Northern Ireland Centre for Stratified Medicine, University of Ulster, Altnagelvin Hospital Campus, Derry/Londonderry BT47 6SB, UK.

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Cystic Fibrosis (CF) lung disease is characterised by a chronic and exaggerated inflammation in the airways. Despite recent developments to therapeutically overcome the underlying functional defect in CFTR (cystic fibrosis transmembrane conductance regulator), there is still an unmet need to also normalise the inflammatory response. The prolonged and heightened inflammatory response in CF is in part mediated by a lack of intrinsic downregulation of the pro-inflammatory NF-κB pathway. We have previously identified reduced expression of the NF-κB down-regulator A20 in CF as a key target to normalise the inflammatory response. Here we have used publically available gene array expression data together with ssCMap (statistically significant connections’ map) to successfully predict drugs already licensed for the use in humans to induce A20 mRNA and protein expression and thereby reduce inflammation. The effect of the predicted drugs on A20 and NF-κB expression (mRNA) as well as pro-inflammatory cytokine release (IL-8) in the presence and absence of bacterial LPS was shown in bronchial epithelial cells lines (16HBE14o-, CFBE410-) and in primary nasal epithelial cells (PNECs) from patients with CF (Phe508del homozygous) and non-CF controls. Additionally, the specificity of the drug action on A20 was confirmed using cell lines with TNFAIP3 (A20) knockdown (siRNA). We also show that the A20 inducing effect of ikarugamycin and quercetin is lower in CF derived airway epithelial cells than in non-CF cells.

A20 (TNFAIP3) | NF-kappaB | Connectivity Mapping | Drug repositioning | CF airway inflammation

Introduction

The response to pathogens, recognised by pattern recognition receptors including Toll-like receptors (TLRs), triggers an acute innate immune response that is mediated by transcription factors such as nuclear factor-kappa-light-chain enhancer of B cells (NF-κB). NF-κB activation promotes the transcription of inflammatory mediators in a tightly regulated process. However, in individuals with underlying chronic inflammatory diseases, this regulation is compromised, leading to constitutive NF-κB activation and persistent inflammation (1-3).

The development of new first-in-class medicines is costly (approximately $1.2 billion for a single FDA-approved drug) and takes between 10 and 15 years (4, 5). Many newly developed drugs perform well in the preclinical testing, but fail when tested in humans (6). Thus alternative approaches using predictive models to identify new drugs are needed. Gene expression connectivity mapping (www.broadinstitute.org/cmap/) is an advanced bioinformatics technique to establish the connections among biological states via gene expression profiles/signatures. One major application of connectivity mapping is to identify potential small molecules able to inhibit a disease state or regulate the expression of a small number of genes (7-9). We used an advanced version of connectivity mapping, ssCMap (statistically significant connections’ map) (10), which has been successfully applied to phenotypic targeting and predicting effective drugs in cancer (10). However, this has not yet been applied to chronic inflammatory diseases.

Cystic Fibrosis (CF) is a chronic multi-organ inflammatory disease, caused by mutations in the CFTR gene (Cystic Fibrosis Transmembrane Conductance Regulator) expressed on apical epithelial surfaces. It is the most common lethal genetic disease in Caucasian populations. Lung disease is the primary cause of morbidity and mortality in CF, resulting from dehydration of epithelial surfaces and reduced mucociliary clearance as a consequence of the ionic imbalance created by CFTR mutation. This leads to a cycle of infection and inflammation associated with a progressive reduction in lung function and eventual respiratory failure. A common feature of CF is the heightened, chronic inflammatory response to Pseudomonas aeruginosa, driven by constitutive NF-κB activation in airway and peripheral blood cells (2, 3, 11). Primary nasal epithelial cells (PNECs) from patients with the common F508del/F508del mutation and a milder genotype (R117H/F508del), show a significant increase in NF-κB(p65) which correlates with disease severity (12).

A20 (TNFAIP3) is a central negative regulator of NF-κB activation following stimulation of TLRs and/or TNF-receptor and regulates different signalling pathways such as NF-κB and interferon regulatory factor (IRF) signalling (13). A20 modifies

Significance

This study reports that publically available gene array expression data together with statistically significant connections’ map (ssCMap) can successfully predict already licensed drugs to modify genes of interest. We applied this bioinformatics approach to the NF-κB regulator A20 (TNFAIP3), which is reduced in Cystic Fibrosis (CF) airway cells. ssCMap predicted drugs that should induce A20 and normalise the inflammatory response in CF. Using airway epithelial cells we show that ikarugamycin and quercetin have anti-inflammatory effects mediated by induction of A20. Using siRNA we confirm that the anti-inflammatory effect of ikarugamycin and quercetin are mainly due to A20 induction. We have identified a process whereby already licensed drugs can be successfully repositioned for chronic inflammatory airway diseases.

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classical immune cells (14, 15) as well as epithelial cells (12), endothelial cells (16), embryonic fibroblasts (17), osteoclasts (18) and pancreatic beta-cells (19) and diverse roles for A20 in innate immunity, apoptosis, autophagy and antigen processing (13, 15, 16, 20). Within the innate inflammatory immune response A20 regulates NF-κB signalling at the level of TRAF6 in mouse embryonic fibroblasts (MEFs) and osteoclasts (17, 18). In cultured human airway epithelial cells, A20 is rapidly induced by viral or bacterial compounds (21) and is essential for termination of the TLR4 signal (22). PNECs stimulated with P. aeruginosa LPS show a transient increase in A20, but CF PNECs display lower A20 expression basally and after LPS stimulation (12, 23).

Therefore, A20 induction should have anti-inflammatory effects within the tightly regulated NF-κB signalling pathway as shown by the induction of A20 through gibberellin (GA3) in airway epithelial cells. GA3 induced A20, reduced IL-8 secretion, stabilised cytosolic IkBα and reduced NF-κB (p65) activation (24). Here we set out to identify additional compounds able to induce A20. Thus, we performed a compound search using gene expression connectivity mapping to identify existing drugs that could induce A20 expression.

Results

1. Connectivity mapping (sscMap):

The selection of gene array data and creation of the gene signature

Data sets that passed the selection criteria contained human primary nasal epithelial cells (PNECs) and the human bronchial epithelial cell lines CFBE410-, Calu-3 and IB3-1 analysed basally and after exposure to P. aeruginosa LPS (Table 1). In total 76 samples from 4 different published gene array data sets were used. Linear expression correlation and GO enrichment analysis for NF-κB pathway genes identified the closest correlates to A20. Table 2 shows the top 7 genes that subsequently served as the input to the connectivity mapping process.

Prediction of drugs to induce A20 in airway epithelial cells

This study sought small molecular compounds that may enhance A20 expression and as a confirming negative control, those compounds that may inhibit A20 expression. Table 3 summarizes the top candidate drugs identified. The column entitled ‘significance’ shows the significance of drugs based on p values and the column ‘z-score’ shows the correlation of the drugs with the input gene signature. Positive z-scores indicate a positive correlation i.e. the input genes are induced when treated with the particular drug. The significant drugs with the highest positive z-scores along with a negative control were selected for laboratory validation. In addition to p values and z scores, stability of the connections was measured by altering the gene signature and the significance of the connections are given under the column ‘perturbation stability’. Drugs with perturbation stability 1 represent strong connections which remain significant with ‘perturbation’ gene signatures. From these predictions two A20

Table 1.

<table>
<thead>
<tr>
<th>ID of data set</th>
<th>Sample Type</th>
<th>Platform used</th>
<th>Sample size</th>
<th>Published in</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSE2395</td>
<td>Primary nasal epithelial cells (PNECs)</td>
<td>HG-U133A &amp; HG-U133B: GPL96/GPL97</td>
<td>100 (18 CF, 112 controls), exposed to P. aeruginosa</td>
<td>Wright JM et al. 2006</td>
</tr>
<tr>
<td>GSE620</td>
<td>IB3-1</td>
<td>HG-U133A: GPL96</td>
<td>5 (IB3-1cells, excluded those with 4-phenylbutyrate treatment)</td>
<td>Wright JM et al. 2004</td>
</tr>
<tr>
<td>GSE923</td>
<td>Calu-3</td>
<td>HG-U133A: GPL96</td>
<td>19 (Calu-3, F508del/F508del infected with P. aeruginosa (mucoid vs. motile strain)</td>
<td>Cobb LM et al. J Immunol 2004</td>
</tr>
</tbody>
</table>

Table 2.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Unique ID</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFAIP3</td>
<td>202643_at</td>
<td>A20</td>
<td>Ubiquitination, negative regulator of NF-κB</td>
</tr>
<tr>
<td>ATF3</td>
<td>202672_at</td>
<td>Activating Transcription Factor 3</td>
<td>Binds the cAMP response element (CRE), transcriptional repressor (promoters with ATF sites)</td>
</tr>
<tr>
<td>RAB5C</td>
<td>201140_at</td>
<td>RAB5C</td>
<td>small GTPase, regulates membrane traffic from plasma membrane to early endosomes, ubiquitously expressed</td>
</tr>
<tr>
<td>DENND4A</td>
<td>214787_at</td>
<td>DENN/MADD domain containing 4A</td>
<td>C-myc promoter-binding protein, promotes exchange of GDP to GTP, converting inactive GDP-bound Rab proteins into their active GTP-bound form</td>
</tr>
</tbody>
</table>
Fig. 1. Gene expression profile of the gene signature genes associated with A20. 16HBE14o- (grey) and CFBE41o- (green) were stimulated (LPS, 10 μg/ml 0-24 h) and mRNA levels of A20, ATF3, Rab5c, DENND4A, POM121, ICAM-1 and PSNE1 determined as described. * significant difference compared to medium control, ** significant differences between genotypes.

16HBE14o- and CFBE41o- were stimulated (LPS, 10 μg/ml 0-24 h) and mRNA levels of A20, ATF3, Rab5c, DENND4A, POM121, ICAM-1 and PSNE1 determined as described. * significant difference compared to medium control, ** significant differences between genotypes.

Fig. 2. A20 and p65 mRNA expression of ikarugamycin, quercetin and fluvastatin treated cells. 16HBE14o- and CFBE41o- were pre-incubated with (a) ikarugamycin, (0.01, 1 μM), (b) quercetin (0.1, 100 μM) or (c) fluvastatin (0.1, 1 μM) stimulated (LPS, 10 μg/ml, 0-24 h), A20 mRNA determined (qRT-PCR) and expressed as A20/β-actin relative to the internal control.

Fig. 3. A20 and p65 mRNA expression of 16HBE14o- and CFBE41o- cultured in the presence or absence of LPS for 0-24h. (Figure 1).

Expression of the genes identified as the A20/NF-kB gene signature in CF epithelial cells were analysed by qRT-PCR in 16HBE14o- and CFBE41o- cultured in the presence or absence of LPS for 0-24h (Figure 1).

Basal expression: CFBE41o- show significantly lower mRNA expression for A20, ATF3, Rab5c and ICAM1 compared to 16HBE14o- (all p<0.05, n=5). Expression of DENND4A and PSNE1 was also lower in CFBE41o- but this did not reach significance.

LPS induced expression: In 16HBE14o-, A20 mRNA is rapidly induced with expression peaking 1h after LPS exposure (p<0.001 compared to medium, n=5), while CFBE41o- show significantly lower (at 1h p<0.001 vs. 16HBE14o-, n=5) and delayed (maximal induction at 4-8 h, p<0.01 and 0.001 vs. medium, n=5) induction upon LPS stimulation. After LPS, ATF3 and ICAM1 expression was significantly lower in CFBE41o- compared to 16HBE14o- (p<0.01, n=5). Pom121 and PSNE1 expression increased in CFBE41o- compared to medium (8h, p<0.05 and p<0.01) and in CFBE41o- compared to 16HBE14o- (8h, p<0.05 and p<0.001). 16HBE14o- showed a significant reduction in DENND4A and Rab5c expression compared to medium (1h, p<0.05 and 4h, p<0.05, respectively), but there was no significant change in the expression of these genes in CFBE41o-.

3. Effect of A20 inducing drugs on cell lines

LDH release in drug exposed 16HBE14o- and CFBE41o-: LDH release was measured after exposure to the drugs alone (0.01–1000 μM) and with LPS stimulation (supplement Figure 1S). Quercetin did not cause any LDH release. Overall fluvastatin was almost without effect on LDH; the exceptions were a slight but statistically significant increase at 10 μM alone (CFBE41o-) and in the presence of LPS (in CFBE41o- and 16HBE14o-). Ikarugamycin (1, 100 μM) caused a significantly higher LDH release in both cell types. In LPS stimulated cells, 0.1 μM ikarugamycin and higher concentrations showed a higher LDH release compared to LPS alone, but this did not reach statistical significance (supplement Figure 1S).
Effect of ikarugamycin, quercetin and fluvastatin on A20 and p65 protein expression. 16HBE14o- and CFBE41o- were pre-incubated with (a) ikarugamycin (0.01, 1 µM), (b) quercetin (0.1, 100 µM) or (c) fluvastatin (0.1, 1 µM) and stimulated (LPS, 10 µg/ml, 0-24 h). Cytosolic A20 and p65 protein was determined by Western Blotting: 1 = Ctr, 2 = LPS, 3 = drug at lower conc., 4 = drug at lower conc. + LPS, 5 = drug at higher conc., 6 = drug at higher conc. + LPS.

LPS stimulated IL-8 release in drug pre-treated 16HBE14o- and CFBE41o-: To assess the anti-inflammatory potential of the selected drugs, cells were pre-treated with the drug for 1h, stimulated with LPS and the IL-8 release measured and the relative IC_{50} calculated (supplement S3). In 16HBE14o-, all drugs reduced IL-8 release by at least 50% with an IC_{50} of 15.6 µM for ikarugamycin, 0.09 µM for quercetin and 0.11 µM for fluvastatin. In CFBE41o-, only quercetin (IC_{50} 0.03 µM) and fluvastatin (IC_{50} 0.001 µM) pre-treatment were able to reduce release by 50%. In contrast, pre-treatment of CFBE41o- with 1 µM ikarugamycin caused a significant increase in IL-8 release compared to LPS alone (LPS 269.9 ± 47.9 pg/ml vs. 590.7 ± 82.6 pg/ml, p<0.05, n=5). Therefore, a meaningful calculation of the relative IC_{50} for IL-8 release in ikarugamycin treated CFBE41o- cells was not possible.

A20 mRNA induction in drug treated 16HBE14o- and CFBE41o-: To elucidate if ikarugamycin and quercetin facilitate their anti-inflammatory action though the induction of A20 as predicted, A20 mRNA was determined by qRT-PCR. Fluvastatin was included as a negative control. Using the LDH and IL-8 release data two drug concentrations were selected for further investigations (supplement S3). In 16HBE14o-, LPS stimulation caused a significant induction of A20 1h after stimulation (Figure 2). Ikarugamycin (0.01 µM) alone did not cause a significant induction of A20, but additional LPS stimulation caused a significant A20 induction at 24h (p<0.05 vs LPS 24h). 1 µM ikarugamycin significantly induced A20 at 4h on its own, but the higher A20 expression in the presence of LPS (1-24h) did not reach statistical significance. Quercetin pre-treatment did not induce significant levels of A20 mRNA at 0.1 µM, alone or in the presence of LPS. However, at 100 µM quercetin A20 mRNA was significantly induced alone at 1h and 4h (p<0.05 vs. medium 1h and 4h) and in the presence of LPS at 4h (p<0.05 vs. LPS 4h). Fluvastatin alone did not induce A20 mRNA at any time or concentration (Figure 2), in the presence of LPS fluvastatin pre-treatment caused a significant reduction in A20 mRNA at both concentrations tested (p<0.001 for 0.1 µM+LPS 1h vs. LPS 1h; p<0.05 for 1 µM+LPS).

The anti-inflammatory effect of ikarugamycin and quercetin is mediated by A20 induction. 16HBE14o- with and without knock-down of A20 were pre-incubated with ikarugamycin, (1 µM) or quercetin (100 µM), stimulated and IL-8 analysed. Drug treatment caused a significant reduction in IL-8 (p<0.05) compared to LPS stimulation alone. A20 knock-down resulted in a lack of the anti-inflammatory effect of the drug tested.
Effect of ikarugamycin, quercetin and fluvastatin on IL-8 release from PNECs from (a) healthy controls and (b) patients with CF. The release of IL-8 (pg/ml) was determined using a commercially available IL-8 ELISA kit. Statistical analysis was performed using Wilcoxon paired ranked t-test.

1h vs. LPS 1h). In CFBE41o-, LPS significantly induced A20 at 4h (p<0.05), but induction levels were lower than in 16HBE14o- (Figure 2). Ikarugamycin (1 μM) induced significant levels of A20 at 4h and 24h (both p<0.05), this was further increased in the presence of LPS at 4h (p<0.05 vs LPS 4h) and 24h (p<0.01 vs LPS 24h). Quercetin treatment alone significantly induced A20 at 4h at both concentrations (p<0.05 0.1 μM vs. medium 4h and p<0.01 100 μM vs. medium 4h). In the presence of LPS, only 100 μM quercetin caused a significant induction of A20 at 24h (p<0.05). Fluvastatin alone did not induce A20 mRNA at any time or concentration (Figure 2), and in the presence of LPS fluvastatin caused a significant reduction in A20 mRNA at both concentrations tested (p<0.05 for 0.1 μM+LPS 4h vs. LPS 4h; p<0.05 for 1 μM+LPS 4h vs. LPS 4h).

**Effect of selected components on NF-κB (p65) mRNA in 16HBE14o- and CFBE41o-**: Next we investigated if A20 induction altered NF-κB(p65) mRNA levels (Figure 2). LPS stimulation caused a significant induction of p65 1h after stimulation in 16HBE14o- (p<0.01 – p<0.001 vs. medium). Ikarugamycin (0.01 μM) was without significant effect on p65 mRNA. At 1 μM, ikarugamycin alone induced p65 at 24h (p<0.01 vs. medium 24h) and in the presence of LPS at 1h and 4h (both p<0.05 vs. LPS). Quercetin alone showed no effect on p65 mRNA levels, but when stimulated with LPS both concentrations of quercetin (0.1, 100
Fig. 6. Effect of ikarugamycin, quercetin and fluvastatin on A20 and p65 mRNA expression in PNECs from (a) healthy controls and (b) patients with CF. Cells were pre-incubated with ikarugamycin, quercetin or fluvastatin at the indicated concentrations (0.01–100 μM) and then stimulated (LPS, 10 μg/ml, 0–24 h). A20 mRNA was determined by qRT-PCR and expressed as A20/β-actin relative to internal control.

Table 1: Selected GEO gene expression data sets selected for the connectivity mapping process. Table 2: Gene expression profile of NF-κB/A20 related genes in CF airway disease. Genes behaving in a similar way than the target gene A20 were determined using linear correlation analyses of the selected gene expression data sets.

<table>
<thead>
<tr>
<th>Drug</th>
<th>C-score</th>
<th>P-value</th>
<th>Z-score</th>
<th>Significance</th>
<th>Perturbation stability</th>
<th>Drug class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azacyclonol</td>
<td>0.3496</td>
<td>1.00E-05</td>
<td>4.8417</td>
<td>1</td>
<td>1</td>
<td>y-Pipradol, ataractive drug</td>
</tr>
<tr>
<td>Ikarugamycin</td>
<td>0.4115</td>
<td>6.00E-05</td>
<td>4.1275</td>
<td>1</td>
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<td>Macrolide type</td>
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<tr>
<td>Quercetin</td>
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<td>3.8963</td>
<td>1</td>
<td>1</td>
<td>Flavenoid</td>
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<tr>
<td>Karakoline</td>
<td>0.4314</td>
<td>1.00E-05</td>
<td>3.8033</td>
<td>1</td>
<td>1</td>
<td>Alkaloid Diterpenoid</td>
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<tr>
<td>Fluvastatin</td>
<td>-0.3532</td>
<td>1.00E-04</td>
<td>-3.8267</td>
<td>1</td>
<td>1</td>
<td>HMG-CoA reductase inhibitor</td>
</tr>
</tbody>
</table>

μM) significantly reduced p65 mRNA levels at 1h (p<0.01 vs LPS 1h). Similar to quercetin, fluvastatin alone showed no effect.
on p65 mRNA levels, but after LPS stimulation fluvastatin (0.1, 100 μM) significantly reduced IL-8 release (p<0.01 vs. medium 4h). Quercetin at 0.01 μM caused a significant induction of A20 compared to medium control at 1h, 4h and 24h (all p<0.01) with expression levels similar to those induced by LPS (Figure 6) and this was maintained the presence of LPS, with a peak A20 induction at 4h. The higher concentration of quercetin (100 μM) significantly induced A20 mRNA at 1h (p<0.05) and subsequent stimulation with LPS resulted in significantly increased A20 mRNA levels at 4h (p<0.05 vs LPS 4h). Fluvastatin treatment did not induce A20 mRNA expression, but 1μM reduced A20 mRNA at 4h LPS (Figure 6).

In CF PNECs LPS induced A20 mRNA expression was lower than in non-CF PNECs, but in CF PNECs LPS induced significant levels of A20 mRNA 4h after LPS (p<0.05 vs. medium 4h). Ikarugamycin at 0.01 μM (alone and in the presence of LPS) had no effect on A20 mRNA levels (Figure 6) but 1 μM ikarugamycin significantly induced A20 mRNA at 4 and 24h alone (p<0.05 vs medium control) and in the presence of LPS (p<0.05 and p<1h and 4h). Similarly, icarin treatment with the lower concentration (0.01 μM) alone and in the presence of LPS had no effect on A20 mRNA levels (Figure 6), while 100 μM quercetin significantly induced A20 mRNA alone (4h, p<0.05 vs medium control) and additionally above LPS induction (1h and 24h, both p<0.05 vs LPS). Similar to non-CF PNECs, fluvastatin treatment of CF PNECs had no significant effect on A20 mRNA expression levels.

NF-κB (p65) induction in PNECs: PNECs from non-CF control subjects respond to LPS exposure with a significant increase in NF-κB (p65) at 1h and 4h (p<0.001 vs medium control). Thereafter, p65 mRNA expression returns to its corresponding medium control value (Figure 6). PNECs from patients with CF however show the expected high levels of p65 throughout the 24h study (p<0.01 at 1h, p<0.05 at 4h and 24h vs medium control) (Figure 6).

In non-CF PNECs, ikarugamycin treatment alone did not change p65 mRNA levels (vs medium control), but in the presence of LPS p65 mRNA was significantly reduced (p<0.05 at 1h, p<0.01 at 4h vs LPS). Overall the higher concentration of ikarugamycin induced p65 mRNA levels with a significant increase at 4h (p<0.05 vs medium control). However, in the presence of LPS, p65 levels remained not significantly different from those after LPS exposure at 1h and 24h, but were significantly lower compared to LPS alone at 4h (p<0.05) (Figure 6). The lower concentration of 0.01 μM quercetin alone did not change basal p65 mRNA. After subsequent LPS challenge p65 mRNA significantly decreased at 1h (p<0.01 vs LPS), and further increased in a similar manner to LPS alone. However, 100 μM quercetin alone significantly reduced p65 induction at 1h and 4h (p<0.05 and p<0.01 vs. medium control). In the presence of LPS this reduction of p65 mRNA reached statistical significance at 4h and 24h (both p<0.05 vs LPS) (Figure 6). Fluvastatin (0.1 μM) induced p65 at 4h (p<0.05 vs medium 4h), but p65 levels remain significantly lower when LPS is added (p<0.05, vs LPS p<0.01 vs LPS). Similarly, icarin treatment with the lower concentration (0.01 μM) alone and in the presence of LPS had no effect on p65 mRNA levels, but after addition of LPS, p65 mRNA was initially reduced (1h p<0.05 vs LPS) but then induced similarly to LPS alone (Figure 6).

When PNECs from patients with CF were pre-treated with ikarugamycin, p65 levels dropped significantly at 0.01 μM ikarugamycin in the absence or presence of LPS (p<0.05-0.01). At the higher concentration of 1 μM p65 levels appeared lower, but this only reached statistical significance at 1h (p<0.05 vs LPS) (Figure 6). Pre-treatment of CF PNECs with quercetin did not affect p65 levels at either concentration tested, but at
0.1 μM quercetin in the presence of LPS significantly reduced p65 at 24h (p<0.05 vs LPS). Treatment with 100 μM quercetin significantly reduced p65 mRNA in the presence of LPS at all time points (all p<0.05 vs LPS) (Figure 6). Fluvasatin alone had no significant effect on p65 mRNA levels, but significantly reduced LPS induced p65 at 1h, 4h and 24h (p<0.05 vs. medium 1h, 4h or 24h), while the higher concentration of fluvasatin (1 μM) showed no significant effect on basal or LPS induced p65 mRNA (Figure 6).

Discussion

Airways infection and the subsequent inflammation are deleterious for patients suffering from CF. Current drugs targeting the mutated CFTR (potentiators/correctors) improve expression and function of CFTR on epithelial surfaces and patients showed improved lung function and reduced frequency of pulmonary exacerbations, hospitalization and use of intravenous antibiotics. bu augmented CFTR function failed to reduce inflammatory markers in sputum (e.g. IL-1,-6,-8) (25) and heterogeneous responses to the treatment have been reported (26), suggesting that CFTR correction/potentiation may not directly improve the underlying compromised immune response. The negative NF-κB regulator A20 (TNFAIP3) is reduced in CF airway epithelial cells, basally and after LPS stimulation (23) and is associated with markers of inflammation and decreased lung function (12). A20 silencing increased TRAF6 and NF-κB activity (18), and A20 over-expression had protective effects in airway inflammation in ‘asthmatic mice’ (27), suggesting that A20 augmentation normalises the inflammatory response in the airways.

In order to find agents to induce A20 in CF we employed sscMAP, which has been widely used in drug development to uncover potential new indications for existing drugs as well as predicting side effects (28). Using disease specific publicly available gene array data on CF (primary nasal) epithelial cells. recent(Jan2016)search revealed no further significant published gene array databases were first selected in August 2013, but a recent (Jan 2016) search revealed no further significant published gene array data on CF (primary nasal) epithelial cells. The applied linear regression model (Pearson’s correlation coefficient) is an established robust method to identify the correlates of a known gene expression estimating the strength of a linear relationship between two random normally distributed variables (29). The application of the linear regression model with GO (gene ontology) selection revealed a gene signature of 6 genes additionally to the seed gene A20 (Table 2) and we confirmed that the expression of these genes is similarly reduced in CF epithelial cells, basally and after LPS stimulation (Figure 1). The identified A20 correlates were ATF3, a transcriptional repressor that binds to CAMP response elements (CRE); RABSC, a small ubiquitously expressed GTPase; DEND4A, which encodes the C-Myc Promoter Binding Protein (MBP-1); POM212, a nuclear transmembrane protein and essential component of the nuclear pore complex; ICAMI, a cell surface glycoprotein typically expressed on endothelial and immune cells, especially during inflammation; and PSEN 1 (Presenilin 1), a catalytic component of γ-secretase and a DREAM binding protein. Further descriptions of these genes and their involvements in inflammation can be found in the online appendix. These genes, as a combined gene signature, were then input into the sscMAP process comparing the gene expression of the gene signature with the gene expression in the reference database (www.broadinstitute.org), which was obtained from systematic microarray gene expression profiling. SscMAP predicted a short list of drugs that should modify the expression profile of the gene signature genes, including A20...
predicted drugs was indeed mainly mediated by the induction of A20. We also tested fluvastatin, which was predicted not to affect or reduce A20 gene expression (negative z-score). Although fluvastatin exerted anti-inflammatory effects (IL-8) in both cell lines, our data show that this was not mediated by the induction of A20 (mRNA), clearly confirming the ssMap prediction. In asthma, fluvastatin inhibits eosinophil adhesion to ICAM-1 (39) and fibroblast proliferation (40). Using similar concentrations we did not observe any reduced proliferation. Fluvastatin at a concentration range similar to those we used, reduced basal and LPS-induced IL-8 release from LPS stimulated whole blood cells, with CF cells appearing more sensitive to fluvastatin than control cells (IC50: 19.1 μM in non-CF cells, 4.6 μM in CF blood cells) (41). In isolated LPS-stimulated peripheral blood monocytes from patients with chronic kidney disease fluvastatin had a significant anti-inflammatory effect (IL-8, IL-6) at a concentration range of 0.0001–1 μM (42). Patients with heart transplants receiving 40 mg fluvastatin/day for 4 weeks showed a significant reduction in total cholesterol levels and a maximum blood fluvastatin concentration of 2.11 and 3.77 μM. These studies suggest that we have covered a physiologically relevant range of fluvastatin. However, fluvastatin metabolism may be affected by concomitant therapies, especially substances competing with cytochrome enzymes and in such cases fluvastatin levels may need to be monitored (43). Any reactions with other therapies (as they would appear in patients with CF) were not investigated in our manuscript as they would have been beyond the scope of the study.

Similar to ivermectin, fluvastatin has been described to have pro-apoptotic effects e.g. in human lymphoma cells, human smooth muscle cells and in rat neonatal cardiomyocytes or rat vascular smooth muscle cells (44, 45), mediated through activation of caspase-3, reactive oxygen species and activation of p38 MAPK (44, 55). However, statins, through their inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), but not through induction of A20, may still have therapeutic potential in airway and systemic inflammation in CF (41).

Overall, our study shows that connectivity mapping (sscMap) can predict A20 inducing drugs. Pre-treatment of cells with both ivermectin and quercetin reduces LPS-induced IL-8 secretion by induction of A20. In non-CF PNECs both drugs upregulated A20 and reduced IL-8 and p65 mRNA at lower concentrations than in cell lines. CF PNECs, however, have a reduced and delayed A20 inducing response to LPS and to the tested drugs and a significant A20 induction appears at the higher concentration of the drugs tested, which might be near to the cytotoxic effect. A20 reduces apoptosis (46, 47) and mutational loss of A20 resulted in rapid apoptosis and inflammation in hematopoietic cells (48). We did not determine markers of apoptosis in our study, but the huge increase in A20 mRNA may indicate a possible counteraction to pro-apoptotic changes in response to ilargamycin treatment. Of particular interest, this may indicate a higher susceptibility/sensitivity of CF cells to pro-apoptotic stimuli.

Our study has several limitations. Firstly, for the ssMap process a huge number of gene array samples are required and although the database search gave a high number of initial results, upon detailed inspection several gene array studies could not be included. Connectivity mapping uses gene array data run on Affimetrix platforms and we selected those performed on these platforms. Several published gene array studies were performed in cell lines. However, the majority of samples selected were PNECs (n=40), but we also included data from cell lines. Furthermore, every published dataset has been performed using a specific experimental design with respect to treatments and time points. We selected experiments that used either no stimulation or exposure to Pseudomonas LPS or to Pseudomonas itself. A sample size of 50–100 individual samples is a statistically acceptable sample size to produce an unbiased result and we used 76 individual gene array samples.

Secondly, the reference database was generated using different cell lines: MCF7, HL60, PC3 and SKMel5. Although all of these results were in agreement with other therapies (as they would appear in patients with CF) were not investigated in our manuscript as they would have been beyond the scope of the study.

Summary
To date there is still a need for alternative anti-inflammatory drugs for patients with CF as restoring CFTR function with potentiators and correctors does not directly affect the inherent innate immune defect. The exaggerated inflammatory response is in part due to the lack of the NF-kB regulator A20 and pharmacological induction of A20 is anti-inflammatory. We have shown here that ssMap is a potent tool to predict effective drugs that can modify A20 without totally inhibiting NF-κB. This is particularly important in the clinical setting as pharmacological suppression of inflammation may increase the incidence of infective exacerbations (51). In addition, A20 inducing drugs have to be carefully adjusted as in addition to the A20 induction e.g. similar to ikarigamycin, fluvastatin has been described to have anti-inflammatory effects e.g. in human lymphoma cells, human smooth muscle cells and in rat neonatal cardiomyocytes or rat vascular smooth muscle cells (44, 45), mediated through activation of caspase-3, reactive oxygen species and activation of p38 MAPK (44, 55). However, statins, through their inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), but not through induction of A20, may still have therapeutic potential in airway and systemic inflammation in CF (41).

Materials and Methods
Selection of Gene Array data
A search of PubMed GEO data sets (http://www.ncbi.nlm.nih.gov/geo/) was performed in August 2013 using the search terms “Cystic Fibrosis”, “epithelial cells”, “airways” and “primary cells”. Data sets that passed the search criteria and were compatible to Affymetrix Human Genome U133A Array were selected.

Connectivity Mapping (sscMap)
Gene expression profiles were generated using Affimetrix Gene chip Microarray and the relative expression of treatment vs. control was sorted...
Cells were exposed to the selected drugs (ikarugamycin, quercetin, flavonoid A, all Sigma-Aldrich, SM5088, Q0125, SM50038, 0.01–1000 μM for 0–1000 μM for 1 h prior to LPS stimulation (P. aeruginosa LPS, Sigma-Aldrich, L9143, 10 μg/mL, up to 24 h). Stock solutions of the drugs were kept at -20°C for up to 3 months. To minimise oxidation or degradation of the compounds, the working dilutions were freshly prepared.

**Determination of LPS induced cytokine release (IL-6 and IL-8)**

IL-6 and IL-8 in cell-free culture supernatants were measured by a commercial available ELISA (PeproTech EC Ltd.) according to the manufacturer’s instructions.

**LDH Cytotoxicity assay**

Lactate dehydrogenase (LDH) release into cell culture supernatants was determined using a LDH-Cytotoxicity Assay Kit (BioVision Ltd.) according to the manufacturer’s instructions.

**Statistical analysis**

All data are presented as the means ± SEM. Differences between groups were analysed using the Kruskal-Wallis non-parametric ANOVA with Dunn’s post-test (*p<0.05, **p<0.01 and ***p<0.001). S denotes a significant difference compared to medium control, while * denotes significant differences between groups (CF vs. non-CF, comparing the same time points or LPS vs. treatment + LPS). The logarithmic inhibitor concentration versus the relative IL-8 response achieving a 50% inhibition was calculated as the relative IC50. GraphPad Prism (La Jolla, California) was used to plot graphs and to analyse the data.

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**References**


CoAreductaseinhibitorsinduceapoptosisoflymphomacellsbypromotingROSgeneration